

Long-Range Electrical Contacting of Redox Enzymes by SWCNT Connectors**

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Dedicated to Prof. Helmut Schwarz on the occasion of his 60th birthday.

The combination of biological molecules and novel nanomaterial components is of great importance in the process of developing new nanoscale devices for future biological, medical, and electronic applications.^[1] The electrical contacting of redox enzymes with electrodes is a subject of extensive research over the last decade, with important implications for developing biosensing enzyme electrodes, biofuel cells and bioelectronic systems.^[2–4] Tethering of redox-relay units to enzymes associated with electrodes,^[5–7] the immobilization of enzymes in redox-polymers,^[8] and the reconstitution of apo enzymes on relay-cofactor units associated with electrodes^[9] were reported as means to establish electrical communication between redox proteins and electrodes. Recently, the reconstitution of the apo-flavoenzyme glucose oxidase, GOx, with a single Au nanoparticle functionalized with the flavin adenine dinucleotide (FAD) cofactor was reported.^[10] The assembly of the Au-nanoparticle/GOx biocatalyst on an electrode led to an effective electrically contacted enzyme electrode. Single-walled carbon nanotubes (SWCNTs) exhibit unique structural, mechanical, and electronic properties,^[11–14] and recent studies have demonstrated the use of SWCNTs in nanodevices and sensors.^[15–19] Several research activities have addressed the generation of biomaterial–SWCNT hybrid systems, protein-linked CNT^[20] and nucleic acid-functionalized SWCNT.^[21,22] The oriented assembly of short SWCNT normal to electrode surfaces was accomplished by the covalent attachment of the CNT to the electrode surfaces.^[23–26] This structural alignment of the SWCNTs allows the secondary association of redox-active components to the CNT, and the examination of charge transport through the SWCNT. Herein we wish to report on the structural alignment of the enzyme glucose oxidase, GOx, on electrodes by using SWCNTs as electrical connectors between the enzyme redox centers and the electrode. We demonstrate that the surface-assembled GOx is electrically contacted to the electrode by means of the SWCNTs, which acts as conductive nanoneedles that electrically wire the enzyme redox-active site to the transducer surface. The effect of the length of the SWCNT on

controlling the electrical-communication properties between the enzyme redox center and the electrode is discussed.

SWCNTs (Carbolex, Sigma) were first purified by heating the as-received nanotubes in refluxing 3 M nitric acid for 24 h and then washing the resulting nanotubes with water by using a 0.6 μm polycarbonate membrane filter (Millipore). The purified long SWCNTs were chemically shortened by oxidation in a mixture of concentrated sulfuric and nitric acids (3:1, 98% and 70%, respectively) that was subjected to sonication for 8 h in an ice/water bath. This procedure yields shortened SWCNTs with a broad length distribution and that have terminal carboxyl functionalities. The shortened SWCNTs were purified by dialysis and filtering. The resulting SWCNTs suspension was further stabilized by sonication for 3 minutes in 1 wt% sodium dodecylsulfate (SDS) as surfactant. After some macroscopic particles had settled down, the SWCNTs supernatant dispersion was loaded onto a controlled pore glass (CPG 3000 \AA , MPG) chromatographic column to perform length fractionation of the SWCNTs and finally dialyzed against a 1 wt% Triton X-100 solution. The eluted SWCNTs fractions (ca. 50 fractions) were analyzed by atomic force microscopy (AFM), to determine the length distribution of SWCNTs in each fraction. The average SWCNTs length decreased as the fraction number increased, and the AFM histograms derived from each fraction showed a relative narrow length distribution. Previous studies have shown that the chemical shortening of SWCNTs by strong acids leads to the formation of carboxylic (and phenolic) groups at the nanotube ends (and sidewall defect sites),^[27] thus allowing the covalent immobilization of the SWCNTs on surfaces. A 2-thioethanol/cystamine mixed monolayer (3:1 ratio) was assembled on an Au electrode and the length fractionalized SWCNTs were coupled to the surface in the presence of the coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as depicted in Figure 1. The incorporation of 2-thioethanol in the mixed monolayer was anticipated to prevent nonspecific adsorption of the surfactant-protected SWCNTs onto the electrode surface (and presumably to prevent lying of the SWCNT pipes on the surface). Figure 2 shows the AFM images of the 50 nm SWCNTs-modified surfaces upon coupling the CNTs to the modified surface for different time-intervals. Longer coupling times lead to higher surface coverage of the SWCNTs. As illustrated in Figure 2A, isolated needlelike protrusions are clearly seen on the surface after 30 minutes of surface modification, the density of which increases gradually, until a densely packed, needlelike pattern of standing SWCNTs is obtained after five hours of coupling. We could not find by AFM measurements SWCNTs that lie on the surface. There could be as many as eight carboxy groups at each end of the 1.3 nm diameter SWCNT of a (16,0) zigzag structure. Therefore, eight amide bonds could be created between each nanotube and the gold surface, thus leading to a preferred standing conformation of the SWCNTs onto the surface. We found, that the height of the surface-standing nanotubes (determined by AFM) relates directly to the length of the corresponding SWCNTs fraction, although the height measured by AFM is always lower than the length of the free SWCNTs adsorbed on mica surfaces and measured by AFM,

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[**] This work is supported by the German-Israeli Program (DIP).
SWCNT = single-walled carbon nanotube.

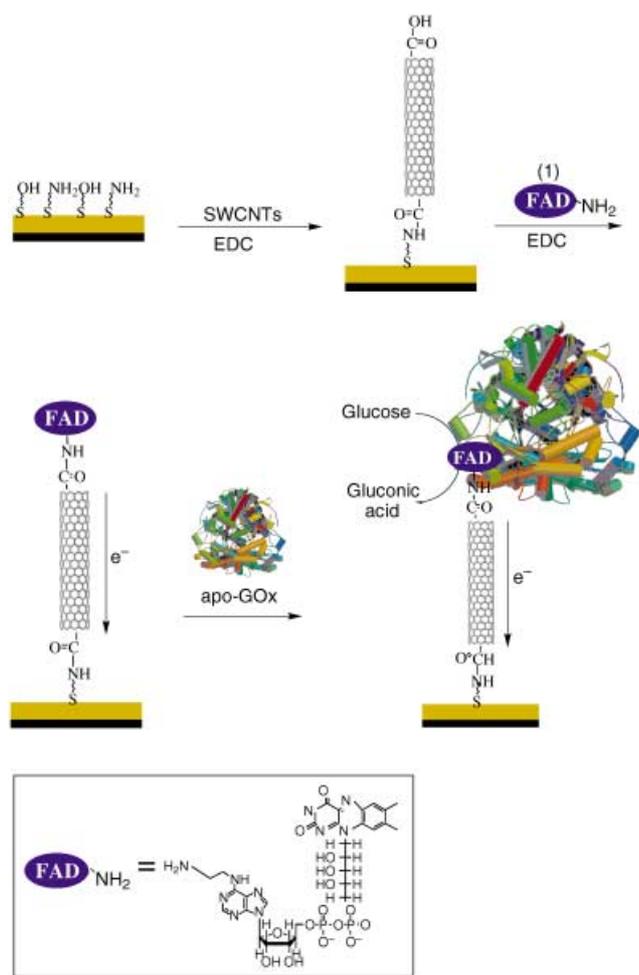


Figure 1. Assembly of the SWCNT electrically contacted glucose oxidase electrode.

Figure 2D. This could be attributed to the fact that the accurate AFM determination of the average SWCNT height is limited by the nature of the SWCNTs assembly. Experiments with a quartz crystal microbalance (QCM) were performed to elucidate the surface coverage of the SWCNTs. From the average length of the SWCNT fractions, the frequency changes observed upon the coupling of the SWCNTs, and the respective mass changes that occurred on the Au/quartz crystal, the surface coverage of the SWCNTs on the surface were calculated (Figure 3). The amino derivative of the FAD cofactor (1), was then coupled to the carboxy groups at the free edges of the standing SWCNTs (after wall protection in the presence of surfactants Triton X-100 and PEG, $\bar{M}_w = 10000$). Cyclic-voltammetry experiments revealed that FAD units were electrically connected with the electrode surface. The FAD units linked to the CNTs reveal a quasireversible cyclic voltammogram, $E^0 = -0.45$ V versus a saturated calomel electrode (SCE) pH 7.4. Coulometric assay of the FAD redox wave and microgravimetric QCM experiments indicate an average surface coverage of about 1.5×10^{-10} mol cm $^{-2}$. Apo-glucose oxidase, apo-GOx, was then reconstituted on the FAD units linked to the ends of the standing SWCNTs. The reconstitution of the apo-GOx units

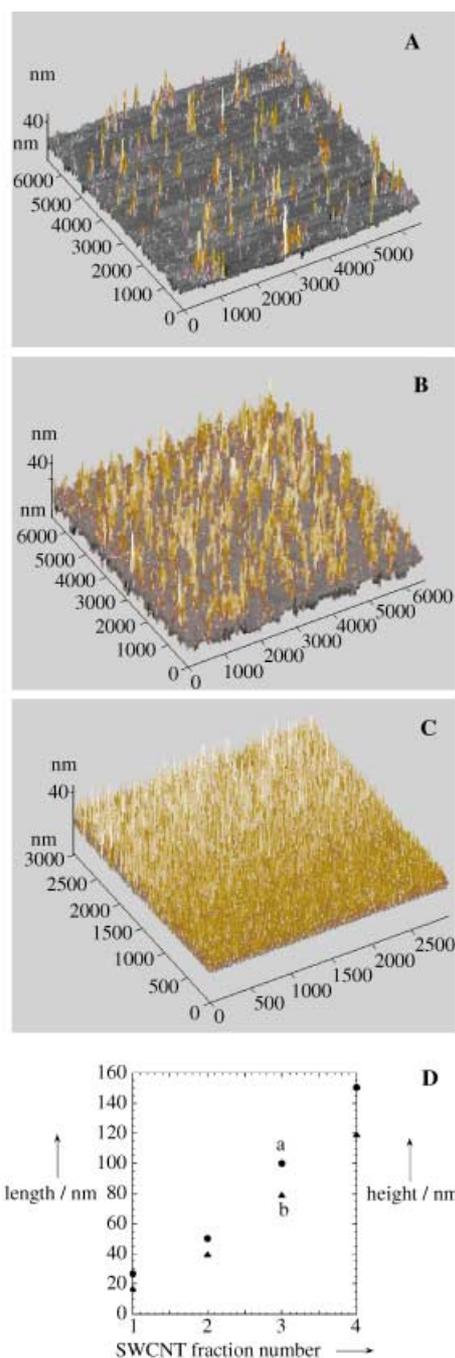


Figure 2. AFM images of SWCNTs covalently linked to a cystamine/2-thioethanol monolayer associated with an Au electrode: A) After 30 minutes of coupling; B) after 90 minutes of coupling; C) after 180 minutes of coupling. D) Comparison of the SWCNTs lengths measured by AFM after deposition on mica surfaces (left ordinate), and the SWCNTs heights measured by AFM (right ordinate) for the different fractions of CNTs.

on the FAD units linked to the ends of the SWCNTs was supported by AFM measurements (Figure 4). In contrast to the SWCNTs needlelike pattern shown in Figure 2, after the enzyme reconstitution, the surface was covered by densely packed clumps with an average lateral dimension of about 5 nm, attributed to individual GOx molecules associated with

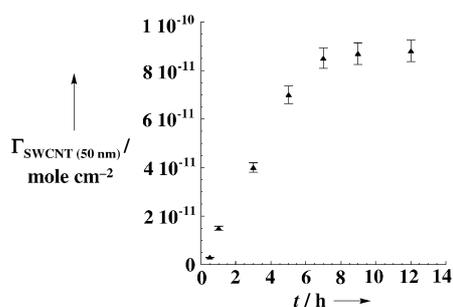


Figure 3. Surface coverage, Γ_{SWCNT} , of the 50 nm long SWCNTs on the Au surface at time intervals of chemical coupling to the surface.

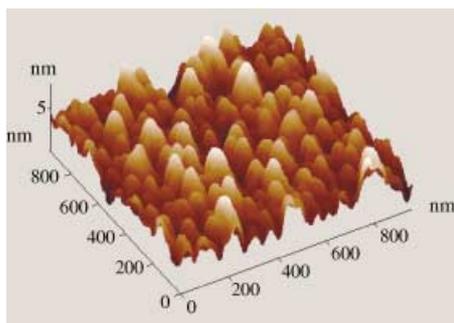


Figure 4. AFM image of the GOx reconstituted on the FAD-functionalized CNTs (ca. 50 nm) monolayer associated with the Au surface.

the SWCNTs. The observed heights of the individual GOx units are consistent with the dimensions of the protein.^[10] The protection of the SWCNTs wall surface prior to the binding of the FAD units and the reconstitution with apo-GOx, is crucial to yield biocatalytic GOx units specifically bound to the free ends of the standing nanotubes. Surfactants such as SDS were shown to be inefficient in preventing nonspecific adsorption of proteins to the CNTs walls.^[20] Indeed, QCM and electrochemical experiments showed that reconstitution of apo-GOx on SDS-protected FAD-modified SWCNTs monolayer linked to the Au-support, leads to the nonspecific adsorption of apo-GOx enzyme units to the walls of the nanotubes. The pretreatment of the SWCNTs monolayer with a mixture of the surfactants Triton X-100 and PEG ($M_w = 10000$) prior to the binding of FAD units and the reconstitution with apo-GOx was found to be an essential step to generate a bioelectrocatalytically active interface, with the enzyme specifically coupled to the SWCNTs FAD-modified ends. Further support that the reconstitution of apo-GOx units takes place preferentially at the edges of the FAD-modified SWCNTs is obtained by analysis of the structures of Triton X-100/PEG protected FAD-modified CNTs reconstituted with GOx in solution (Figure 5). In these experiments, CNTs are functionalized in solution with (1), and reconstituted with apo-GOx. The modified enzyme CNTs were deposited on mica. Figure 5A shows the AFM image of the GOx-SWCNTs hybrid. We observe SWCNTs with one or two enzyme units at the edges of the tubes. The height of the enzyme units is about 5 nm,^[10] which is consistent with the dimensions of GOx. Figure 5B shows the high resolution

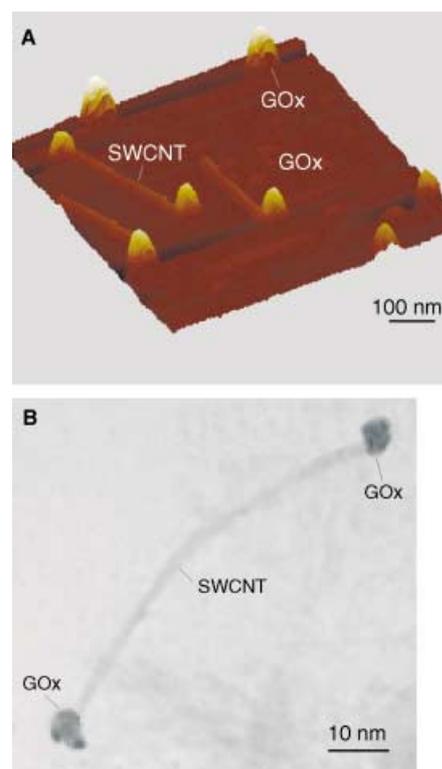


Figure 5. A) AFM image of SWCNTs reconstituted at their ends with GOx units. B) HRTEM image of a SWCNT modified at its ends with GOx units.

TEM image (HRTEM) of a CNT modified with two GOx units (negatively stained with uranyl acetate) at the edges of the tube. Experiments with surfactant-free or SDS protected SWCNTs, showed a great extent of nonspecific adsorption of the protein onto the wall of the carbon nanotubes.

The modification of the surface with the reconstituted GOx units was further characterized by means of microgravimetric QCM and electrochemical experiments. Au/quartz crystals (9 MHz) were modified with a 2-thioethanol/cystamine mixed monolayer, the SWCNTs were then coupled to the surface, FAD units were coupled to the CNTs protected with Triton X-100/PEG, and the apo-GOx was reconstituted on the surface. From the frequency changes of the crystals and the voltammograms of the FAD units, we estimate the surface coverage of the CNTs (using the fraction of about 25 nm average length) and of the GOx units to be $4 \times 10^{-11} \text{ mol cm}^{-2}$ (3–4 FAD units per SWCNT) and $1 \times 10^{-12} \text{ mol cm}^{-2}$, respectively.

Figure 6A shows the cyclic voltammograms corresponding to the GOx-CNT-functionalized Au electrode (average CNT length 25 nm) in the presence of different concentrations of glucose. The bioelectrocatalytic oxidation of glucose is observed at $E > 0.18 \text{ V}$ versus SCE, and the electrocatalytic anodic current becomes higher as the concentration of glucose increases. The respective calibration curve, depicted in Figure 6B, shows a saturation current that corresponds to 60 μA . Knowing the surface coverage of the GOx-CNT units, we estimate the turnover rate of electrons transferred to the electrodes to be about 4100 s^{-1} . This value is about sixfold

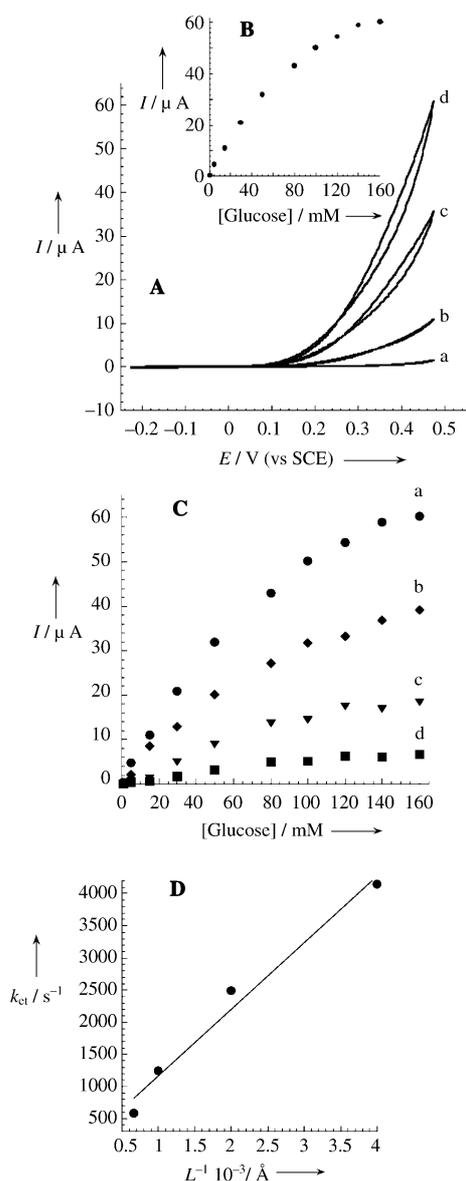


Figure 6. A) Cyclic voltammograms corresponding to the electrocatalyzed oxidation of different concentrations of glucose by the GOx reconstituted on the 25 nm long FAD-functionalized CNTs assembly: a) 0 mM glucose, b) 20 mM glucose, c) 60 mM glucose, d) 160 mM glucose. Data recorded in phosphate buffer, 0.1 M, pH 7.4, scan rate 5 mVs⁻¹. B) Calibration curve corresponding to the amperometric responses of the reconstituted GOx/CNTs (25 nm) electrode (at $E = 0.45$ V) in the presence of different concentrations of glucose. C) Calibration curves corresponding to the amperometric responses (at $E = 0.45$ V) of reconstituted GOx–CNTs electrodes in the presence of variable concentrations of glucose and different CNT lengths as electrical connector units: a) about 25 nm SWCNTs, b) about 50 nm SWCNTs, c) about 100 nm SWCNTs, d) about 150 nm SWCNTs. D) Dependence of the electron-transfer turnover rate between the GOx redox center and the electrode on the lengths of the SWCNTs comprising the enzyme electrodes.

higher than the turnover rate of electrons from the active site of GOx to its natural O₂ electron acceptor (700 s⁻¹).^[28] Figure 6C shows the calibration curves corresponding to the anodic currents generated by GOx-reconstituted SWCNT

electrodes of different SWCNT lengths, in the presence of variable glucose concentrations (the currents were normalized to the same GOx surface coverage determined by QCM). A clear dependence on the length of the CNTs is observed. The GOx–CNT with an average length of 25 nm reveals an about 1.5-fold enhanced electrocatalytic current (or electrical communication) as compared to the GOx–CNT electrode with an average length of 50 nm (2570 s⁻¹). Thus, the electron-transfer barrier between the FAD center and the electrode, is lower for systems that include shorter SWCNTs as connectors. This length-controlled electron transfer is further supported by examining the interfacial electron transfer to the FAD sites at the ends of the standing SWCNTs. The interfacial electron-transfer rate constants, between the FAD sites and the electrode, in the presence of SWCNTs of different lengths, before the reconstitution of the enzyme apo–GOx, were extracted by the electrochemical Laviron analysis.^[29] From this analysis, we estimate the interfacial electron-transfer rate constants to be 83 s⁻¹, 42 s⁻¹, 19 s⁻¹, and 12 s⁻¹, for assemblies that include standing SWCNTs of 25 nm, 50 nm, 100 nm, and 150 nm average length, respectively. Thus, the nanotube length indeed controls the electron transfer between the FAD units and the electrode, a process that is reflected in the overall bioelectrocatalytic oxidation of glucose. Although the mechanism of CNT length-controlled electrical contacting of the enzyme redox center and the electrode is, at present, not fully understood, these results clearly indicate that electrons are transported through the SWCNT along distances greater than 150 nm from the enzymatic active center to the electrode. These distances eliminate the possibility of charge transport by tunneling processes. Our results reveal, however, that the electrical communication is controlled by the length of the CNTs. A possible origin for the length-dependent electrical communication, found here, may be the fact that wall defect sites are introduced into the CNT walls upon their oxidative shortening. Such sites may act as local barriers to charge transport. Back scattering of the transported electrons or the electron hopping over these defect sites may then explain the length dependence. Figure 6D shows the linear dependence between the turnover rate of electron transfer and L^{-1} (L is the CNT length). This linear dependence suggests that the back scattering or electron hopping prevail in charge transport through SWCNTs and the electrical activation of the biocatalyst.

To conclude, our study demonstrates for the first time the aligned reconstitution of a redox flavoenzyme (glucose oxidase) on the edge of carbon nanotubes that are linked to an electrode surface. We reveal that the SWCNT acts as a nanoconnector that electrically contacts the active site of the enzyme and the electrode. The electrons are transported along distances greater than 150 nm and the rate of electron transport is controlled by the length of the SWCNTs. These results show the compatibility of SWCNTs with the preparation of novel biomaterial hybrid systems that may have fascinating new properties.

Received: November 6, 2003 [Z53275]

Keywords: biosensors · electrochemistry · enzymes · nanotechnology · nanotubes

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