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 apo enzymes on relay-cofactor units associated with electrodes[8] 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.[9] The assembly of the Au-nanoparticle/GOx biocatalyst on an electrode led to an effective electrically contacted enzyme assembly of the Au-nanoparticle/GOx biocatalyst on an 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 Å, 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 SWCNTs adsorbed on mica surfaces and measured by AFM.


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Figure 2D. This could be attributed to the fact that the accurate AFM determination of the average SWCNT heigth 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, \(M_w = 10,000\)). 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, \(E^o = -0.45\) 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 \(1.5 \times 10^{-10}\) 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 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
the SWCNTs. The observed heights of the individual GOx units are consistent with the dimensions of the protein.\textsuperscript{[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.\textsuperscript{[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 were functionalized in solution with (I), 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,\textsuperscript{[10]} which is consistent with the dimensions of GOx. Figure 5B shows the high resolution 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 $4 \times 10^{-11}$ mol cm$^{-2}$ (3–4 FAD units per SWCNT) and $1 \times 10^{-12}$ 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 $E > 0.18$ 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 $\mu$A. 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.
higher than the turnover rate of electrons from the active site of GOx to its natural O₂ electron acceptor (700 s⁻¹).²⁰

Figure 6C shows the calibration curves corresponding to the amperometric responses (at $E = 0.45 \text{ V}$) of reconstituted GOx–CNT 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.

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