Specific Vectorial Immobilization of Oligonucleotide-Modified Yeast Cytochrome c on Carbon Nanotubes

Hendrik A. Heering,*[a, b] Keith A. Williams,[b] Simon de Vries,[c] and Cees Dekker[b]

Iso-1-cytochrome c from the yeast Saccharomyces cerevisiae (YCC) contains a surface cysteine residue, Cys102, that is located opposite to the lysine-rich side containing the exposed heme edge, which is the docking site for enzymes. Site-specific vectorial immobilization of YCC via Cys102 on single-walled carbon nanotubes (SWNT) thus provides a selective interface between nanoscopic electronic devices and complex enzymes. We have achieved this by modification of Cys102 with an oligonucleotide (dT18). Atomic force microscopy, fluorescence imaging, and cyclic voltammetry show the specific adsorption of YCC, modified with dT18, on the SWNT sidewall with retention of its native properties. Pretreatment of the SWNT with Triton-X405 blocks the nonspecific binding of untreated YCC but does not interfere with binding of the oligonucleotide-modified YCC.

Introduction

Carbon nanotubes are important materials for the fabrication of nanoscopic electrodes. Single-walled carbon nanotubes (SWNTs) have a well-defined cylindrical geometry, with a diameter in the nanometer range.1 Recently, Heller et al.2 demonstrated that individual SWNTs can be used as nanoelectrodes for electrochemistry. Devices were fabricated in which ≈1 μm of a SWNT sidewall was exposed to an aqueous solution of a ferrocene derivative, and cyclic voltammetry was performed. The nanoscopic dimensions led to a high current density at the electrode surface, allowing the study of both the fast heterogeneous electron-transfer kinetics at the nanotube sidewall, and redox enzyme kinetics that are not limited by mass transport. Advances in the bio-functionalization of SWNTs open a route towards electrochemical single-biomolecule studies.3–11 The high conductivity pathway provided by SWNT has been successfully applied in macroscopic enzyme electrodes consist-ing of a vertically aligned carbon nanotube “forest”.12–7 Nano-scopic electrodes made from an individually contacted SWNT may allow innovative biological applications, such as the probing of local cellular environments and, ultimately, the measurement of the electrocatalytic activity of a single redox enzyme.

Since very few biomolecules—or even just a single one—may be present on such a small electrode, a strong, efficient, and (preferably) an orientationally specific immobilization of the biomolecules is of paramount importance. The durability of the device may be improved by optimizing the orientation of the enzyme for stability. Moreover, the vectorial immobiliza-tion of a redox enzyme is important to obtain a fast and well-defined electron-transfer pathway. This can be achieved by coupling a unique surface amino-acid residue, either directly or via a short spacer, to the electrode surface. Alternatively, not the enzyme itself but instead its natural redox partner protein can be adsorbed, provided that the docking site for the enzyme is facing the solution. Because many enzymes exchange electrons with small redox proteins, such as azurin, ferredoxin, and cytochrome c, such a device will have a broad applicability.

Mitochondrial cytochrome c has been shown to spontaneously adsorb on carbon nanotubes.12–14 The interaction most likely occurs via the protein side that is rich in Lys residues, since the primary amines strongly bind to carboxylate functionalities,15–19 and amines are also known to strongly interact with the carbon-nanotube sidewall.20–22 Because the heme group slightly protrudes into the solution on that side, this ori-entation is very favorable for fast electron exchange. Unfortu-nately, the same Lys-rich side of cytochrome c is also the docking site for enzymes.23–28 Such nonspecific adsorption will therefore hamper the interaction with the redox enzymes and thereby severely limit its utility. The application of cytochrome c as an intermediate between a carbon nanotube and a redox enzyme thus requires the modification of an amino-acid resi-
due opposite to the heme cleft. Fortunately, iso-1-cytochrome c from the yeast Saccharomyces cerevisiae (YCC) contains a unique, naturally occurring surface cysteine residue. Notably, this Cys102 is located opposite to the lysine-rich side containing the exposed edge of the heme.[29] The properties of vectorially oriented YCC, chemisorbed via Cys102 at thiol-modified surfaces, have been extensively studied.[30–33] Scanning probe microscopy has shown that YCC can be immobilized directly on gold via Cys102.[34–36] Moreover, we have shown that YCC, vectorially chemisorbed on gold, exhibits very fast, reversible interfacial electron transfer and retains its native functionality, that is, the ability to interact with redox enzymes in solution. Electron relay via YCC is sufficiently fast to reveal mechanistic properties of these enzymes.[37–39] The goal herein is to develop a method to vectorially immobilize YCC on a carbon-nanotube sidewall without introducing defects that would reduce the conductivity. The method will be suitable for applications to nanofabricated devices in which the electrode consists of a partially exposed SWNT. This will enable the fabrication of a biocompatible nanoscopic electrode with specific docking sites for a variety of unmodified redox enzymes. We demonstrate that YCC can be tethered to the sidewall of a SWNT via Cys102 with retention of its native properties. To achieve this, we modify the Cys102 thiol with an oligonucleotide and utilize the ability of oligonucleotides to form a strong, noncovalent complex with the sidewall of a carbon nanotube.[37] The addition of the surfactant Triton-X405 prevents the nonspecific adsorption of YCC.

**Experimental Section**

Materials: Iso-1-cytochrome c from YCC was purchased from Sigma. The phosphate buffer saline (PBS) consists of a solution of sodium phosphate (NaH₂PO₄ and Na₂HPO₃, from Merck; 20 mM total phosphate) and NaCl (150 mM) at pH 7. Heps buffer [N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid), Sigma] was titrated to pH 7 with NaOH. The surfactant Triton-X405 was added from a 10% stock solution (Aldrich). Aqueous suspensions of carbon nanotube.[37] The addition of the surfactant Triton-X405 prevents the nonspecific adsorption of YCC. A solution of YCC (18 µM) in PBS was then incubated for one hour with the modified YCC in 20 mM Hepes with 0.5 mM MgSO₄, pH 7.) was separated from the product (Cy3–YCC–dT₁₈) by repeated dilution with PBS and concentration over a 10 kDa cutoff filter (Microcon YM10, Amicon), as described above, until the filtrate was free from oligonucleotide. To prevent nonspecific binding of the oligonucleotide to YCC, 0.5 mM MgSO₄, (J. T. Baker) was added to the PBS buffer.

A portion of the dT₁₈-modified YCC (YCC–dT₁₈) was fluorescently labeled by incubating YCC–dT₁₈ (10 µM) in PBS for one hour with the mono-succinimidyl ester of Cy3 (Cy3, Amersham Biosciences; 50 µM added from a 5 mM stock solution in DMSO). Unreacted Cy3 was separated from the product (Cy3–YCC–dT₁₈) by repeated dilution in PBS and concentration over a YM10 filter, as described above, until the filtrate was free from Cy3. The concentrations of cytochrome c, oligonucleotide, and Cy3, as well as the yield and integrity of the products, were determined by UV/Vis absorption spectroscopy (Jasco V-530 spectrophotometer).

Atomic Force Microscopy (AFM): The SWNTs were grown through chemical vapor deposition (CVD) from catalyst islands on a silicon wafer with a thermally grown oxide layer.[40] These devices are similar to those used by Heller et al. to fabricate SWNT sidewall electrodes.[41] The devices were incubated for 1 to 5 min with YCC or YCC–dT₁₈ in PBS (with or without Triton-X405 in solution) or only with Triton-X405 in PBS, then thoroughly rinsed with water, dried under a stream of nitrogen, and mounted onto the atomic force microscope (Nanoscope IV, Digital Instruments). The images were recorded in the tapping mode, using silica cantilevers with a typical tip size of 10 nm (Olympus).

Fluorescence Microscopy: A suspension of HiPCO SWNTs (0.5 µg/mL) in Triton-X405 (0.05%) and Cy3–YCC–dT₁₈ (0.1 µM) in PBS was incubated for one hour. Freshly cleaved mica (grade V-4, SPI supplies) was incubated with 0.01% poly-L-lysine (5–10 kDa, Fluka) for 20 s and then rinsed with water and dried under a stream of nitrogen. The modified nanotubes were deposited on this surface by applying the suspension for 20 s, followed by rinsing and drying. The samples were examined with a fluorescence microscope (Leica DMRXA) equipped with a spectrum analyzer (SpectraCube, Applied Spectral Imaging). Fluorescent images were obtained using optical filters, with excitation between 515 and 560 nm and detection of the emission above 560 nm. The emission spectrum of the fluorescent features was verified to be that of Cy3. The samples were also inspected with white light for structural features.

Electrochemistry: For measuring the reduction potential of (modified) YCC in solution, a 2 mm² gold disk was used as the working electrode (Bioanalytical Systems). The gold electrode was polished with a water-based diamond suspension (Buehler, 1 µm particles), rinsed, and then incubated for 30 min in 10 mM 6-mercaptop-1-hexanol (Aldrich). A 10–25 µL droplet of solution (10 µM YCC or modified YCC in 20 mM Hepes with 0.5 µM MgSO₄, pH 7) was placed between the working electrode, the reference electrode, and a platinum-wire counter electrode, as described by Hagen.[42] The cell was flushed with wetted nitrogen and connected to a Bioanalytical Systems CV-50W potentiostat for cyclic voltammetry. The reference electrode was a Ag/AgCl/3 M NaCl electrode (Bioanalytical Systems RE-5B, +215 mV versus the normal hydrogen electrode, NHE). All potentials are given versus the NHE. Carbon-nanotube electrodes were prepared from a suspension (≈1 mg/mL) of HiPCO SWNTs in ethanol. A droplet of the suspension was applied on a gold electrode and the solvent evaporated at 150 °C. This was repeated several times to obtain a uniform, dense layer of carbon nanotubes. The nanotube electrodes were then used in the setup described above.
Results and Discussion

Properties of YCC Modified with dT18

Since poly-dT is known to strongly bind to the SWNT sidewall,[37] Cys102 is covalently attached via a short, bifunctional maleimide/succinimidyl linker to a dT18 oligonucleotide containing a 3' amine. The ratio of dT18 to YCC is 1.03, as determined from the increase of the absorbance at 260 nm, using $\varepsilon_{260\text{nm}} = 180.4 \text{mM}^{-1}\text{cm}^{-1}$ for dT18. The UV/Vis spectra in Figure 1 show that the attachment of the dT18 oligonucleotide to Cys102 does not alter the spectroscopic properties of the heme. In addition, the reduction potentials of both unaltered YCC and YCC–dT18, as determined by diffusion-controlled cyclic voltammetry with a mercaptohexanol-modified gold electrode, are both 280 mV at pH 7 (data not shown). This indicates that the modification does not alter the properties of YCC. The UV/Vis spectra and the reduction potentials are also not affected by the addition of 1% Triton-X405.

Atomic Force Microscopy

To prevent nonspecific binding of YCC on the carbon nanotubes, we tested the effect of Triton-X405 on the adsorption of YCC on basal-plane graphite. Preliminary AFM trials showed that YCC does adsorb from a 1 μm solution in PBS on bare basal-plane graphite. However, it adsorbs to a much lesser extent when the surface is pretreated with 1% Triton-X405 (data not shown). This indicates that the modification does not alter the properties of YCC. The UV/Vis spectra and the reduction potentials are also not affected by the addition of 1% Triton-X405.

Fluorescent Labeling

To confirm that the AFM features on top of the carbon nanotubes are indeed YCC molecules we labeled YCC–dT18 with the fluorescent dye Cy3. The ratio of Cy3 to YCC is 1.10, as deter-
mined from the increase of the absorbance at 548 nm, with $e_{548nm} = 196 \text{mm}^{-1} \text{cm}^{-1}$ for reduced YCC, and $e_{548nm} = 150 \text{mm}^{-1} \text{cm}^{-1}$ for Cy3. As shown in Figure 1, the heme Soret band at 413 nm in the UV/Vis absorption spectrum is comparable to that of unmodified YCC. Moreover, the reduction potential of this Cy3–YCC–dT18 construct, measured with cyclic voltammetry at a mercaptohexanol-modified gold electrode, is equal to that of untreated YCC (data not shown). This implies that the double modification of YCC with both Cy3 and dT18 does not significantly alter the electronic properties of the heme.

A suspension of 0.5 mg L$^{-1}$ carbon nanotubes in 0.05% Triton-X405 was incubated for one hour with 0.1 μM Cy3–YCC–dT18 in PBS (which corresponds to one YCC molecule per ≈3 nm of nanotube). These nanotubes were subsequently deposited on a mica surface that was pretreated with poly-lysine to immobilize the carbon nanotubes. Figure 4 shows a fluorescence image of the sample. The distribution of the fluorescent features suggests that the Cy3 labels are assembled on linear structures with a length of >2 μm. This arrangement does not coincide with either mica steps or other features on the surface, as verified by imaging with white backlighting. Incubation of nanotubes and not with the underlying contact electrode. YCC–dT18 can only exchange electrons with the top-most layer of nanotubes and not with the underlying contact electrode. Firstly, the nanotube electrode was incubated with a 1% Triton-X405 solution for 1 min to block nonspecific binding of YCC. Then, it was incubated for 40 min with 0.75 μM YCC–dT18 in PBS and 0.1% Triton-X405, thoroughly rinsed, and measured in PBS with 1 m MgSO$_4$ (pH 7.0).

As shown in Figure 5A, a clear and reversible pair of peaks is observed at 269 (cathodic) and 289 mV (anodic) after incubation with YCC–dT18. The integral of the peaks is 0.2 ± 0.04 μC, which corresponds to 2 pmol of adsorbed YCC. Considering the crystallographic dimensions of YCC,[29] this occupies a surface area of 11 nm$^2$, which is equivalent to a nearly full electroactive coverage on the upper-layer nanotubes (accounting for surface roughness). The background current of 11.2 μA at 50 mV s$^{-1}$ yields a capacitance of 32 F m$^{-2}$, compared to 0.9 F m$^{-2}$ for the bare gold electrode. The relatively large capacitance can be attributed to the larger surface area of the nanotube mesh probed by small ions, compared to that accessible to YCC–dT18. The YCC peaks appear to be narrow for a one-electron reaction, but this is due to the restricted scan range used (140 mV). At a wider range, however, the characteristic carbon-electrode surface peaks at 168 (cathodic) and 316 mV (anodic) obscure the YCC response (Figure 5B). The small separation between the cathodic and anodic YCC peaks is comparable to the finite peak separation (not attributable to electron-transfer kinetics) observed for YCC directly chemisorbed via Cys102 on gold.[36] The midpoint potential of adsorbed YCC–dT18 is 279 mV, which is equal to that of YCC in solution. This implies that YCC–dT18 retains its native conformation, and that the heme group is outside the electric field emanating from the nanotubes. This is consistent with tethering of YCC via Cys102–dT18 to the exposed upper layer of the dense nanotube mesh, with the heme cleft facing the solution.

**Cyclic Voltammetry**

The integrity of the immobilized YCC–dT18 construct and its ability to exchange electrons with the SWNT are assessed by voltammetry. A macroscopic electrode is required for obtaining a large enough YCC sample to yield detectable direct oxidation and reduction currents. We used an electrode consisting of a densely packed multilayer of carbon nanotubes to ensure that YCC–dT18 can only exchange electrons with the top-most layer of nanotubes and not with the underlying contact electrode. We used an electrode consisting of a densely packed multilayer of carbon nanotubes to ensure that YCC–dT18 can only exchange electrons with the top-most layer of nanotubes and not with the underlying contact electrode. Thus, the voltammogram of YCC–dT18 at wide and narrow scan ranges.

**Figure 5.** Voltammograms of YCC–dT18 (1), the recorded background (2), and the difference ([3]; fivefold expanded). B) Comparison of the voltammograms of YCC–dT18 at wide and narrow scan ranges.
Conclusions

We have demonstrated that the sidewalls of the Triton-coated carbon nanotubes can only be decorated with YCC when Cys102 is modified with an oligonucleotide. Triton-X405 effectively prevents the nonspecific adsorption of unmodified YCC on the SWNT, but does not interfere with the oligonucleotide binding. Adsorption is apparent from AFM, as the carbon nanotubes are decorated with features with the expected height of YCC. When the carbon nanotubes are incubated with the spectroscopic properties of YCC. The redox properties are also not affected when this hybrid is bound to carbon nanotubes. Moreover, on a macroscopic carbon-nanotube-multilayer electrode, a voltammetric response is observed at the reduction potential of YCC. Therefore, the AFM features are assigned to the sidewalls of the Triton-coated carbon nanotubes. Thus, cytochrome c can be specifically immobilized on the SWNT with retention of its native properties and in an orientation that allows for electron transfer and favors the interaction with the redox enzymes. Nanoscopic electrodes consisting of one YCC-modified SWNT are a significant step towards single-enzyme electrochemistry and applications in selective nanoscopic biosensor devices.

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References


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