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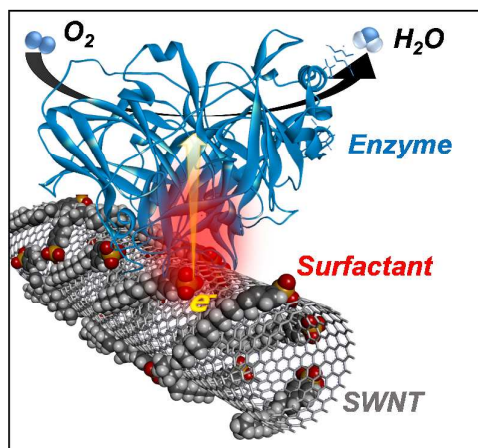
The pre-treatment of carbon nanotube-based nanostructured porous electrodes with surfactants enhanced the efficiency of direct electron transfer of multi-copper oxidases.

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Surfactant-Assisted Direct Electron Transfer between Multi-Copper Oxidases and Carbon Nanotube-Based Porous Electrodes

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The effects of pre-treatment with surfactants on the electrocatalytic reaction of multi-copper oxidases were quantitatively evaluated using a well-structured carbon nanotube forest electrode. It was found that both the charge polarity of the head group and the aromatics in the tail part of the surfactants affect the efficiency of enzymatic electrocatalysis.

Enhancing the efficiency of direct electron transfer (DET) between redox enzymes and electrodes is a key challenge in the development of enzymatic biodevices such as biofuel cells and biosensors.[1-5] Precise studies using smooth electrodes have revealed that appropriate orientation of multi-copper oxidases (MCOs), such as bilirubin oxidase (BOD) and laccase (LAC), result in more efficient DET,[6-8] although the O_2 reduction current density was in the μA cm^{-2} range due to the limited amount of adsorbed enzymes. Practical current densities in the mA cm^{-2} range can be obtained using porous electrodes composed of nanocarbons such as active carbon particles and carbon nanotubes (CNTs) because of their ability to immobilize a large amount of enzymes into their intra-nanospaces.[9-12] To prepare a CNT-based nanostructured electrode, a dispersion of CNTs is usually cast on an electrode support and dried,[13,14] followed by loading of an enzyme solution for immobilization. The efficient utilization of the intra-nanospace of CNT-based electrodes requires the entire penetration of aqueous enzyme solutions into the nanostructures. Therefore, prior to the enzyme loading, the hydrophobic nature of pristine CNT should be changed to hydrophilic by pre-treatment with acids or surfactants. The acid treatment is known to cause the formation of hydrophilic oxygen-containing functional groups on the CNT sidewalls. The surfactant molecules non-covalently adsorb on the CNT sidewalls with their hydrophobic tail and invite the enzyme solutions into the nanostructures through their hydrophilic head groups.

Here, we studied for the first time the effect of presence of surfactants on the DET reaction of BOD and LAC, using electrodes possessing both conventional random CNTs (rCNT) and a carbon nanotube forest (CNTF), the latter of which is a free standing film consisting of long (~ 1 mm) single-walled CNTs. The extremely well-aligned structure of the CNTF enables quantitative, reproducible investigations, including the analysis of the amount of enzyme molecules incorporated inside the nanostructured electrodes. We evaluated the effects of charge polarity of the head-groups of the surfactants and the presence of aromatic groups in the molecular structure of the surfactants.

Bilirubin oxidase (BOD; EC 1.3.3.5, 2.5 U mg^{-1}, from *Myrothecium*, purchased from Amano Enzyme Co.) was used as received. Laccase (LAC; EC 1.10.3.2, 108 U mg^{-1}, from *Trametes* sp, purchased from Daiwa Kasei Co.) was purified by anion exchange chromatography with a DEAE-Toyopearl column.[15] Three kinds of alkyl-chain containing surfactant salts were used as received: sodium dodecyl sulfate (DS), sodium dodecyl benzene sulfonate (DBS), dodecyl aniline (DA).

Single-walled CNT, whose length ranged from 10~30 μm (Sigma), was dispersed in N, N-dimethylformamide (2.5 mg ml^{-1}) and a 5 μL aliquot was cast onto glassy carbon to construct a conventional rCNT electrode, followed by drying at 70°C (Fig. 1a). This rCNT electrode was first treated with 0.1 % surfactant solutions (the three kinds shown in Fig. 1b) and then modified with BOD by dropping 5 μl of phosphate buffered saline (PBS; pH 7.0) containing 1 mg ml^{-1} BOD and drying at room temperature. Fig. 1c shows photographs of water droplets on the conventional rCNT electrodes before and after the treatment with 0.1 wt% DBS. The contact angle of the droplet changed from 136° to 41° by the treatment, indicating the enhanced wettability that is required for effective enzyme loading. Fig. 1d shows typical cyclic voltammograms (CVs) of the BOD/rCNT electrodes pre-treated, or not, with surfactant, measured at 10 mV s^{-1} in a stirred O_2-saturated buffer solution. As expected, the rCNT electrodes treated with all of three kinds of surfactants showed clear catalytic activity for O_2 reduction owing to the penetration of enzyme into the nanostructure of electrodes, whereas the electrode without surfactant (black plot) was almost inert. The kind of surfactant used seem to affect the resulting electrode activity. For example, the modification with DS, which is one of commonly used surfactants, produced activity that was inferior to DBS. However, due to the poor reproducibility and the mechanical fragility of the conventional rCNT electrodes, it is hard to quantitatively compare the effects of surfactants in detail. Therefore, we next conducted experiments using high-quality CNTF electrodes.
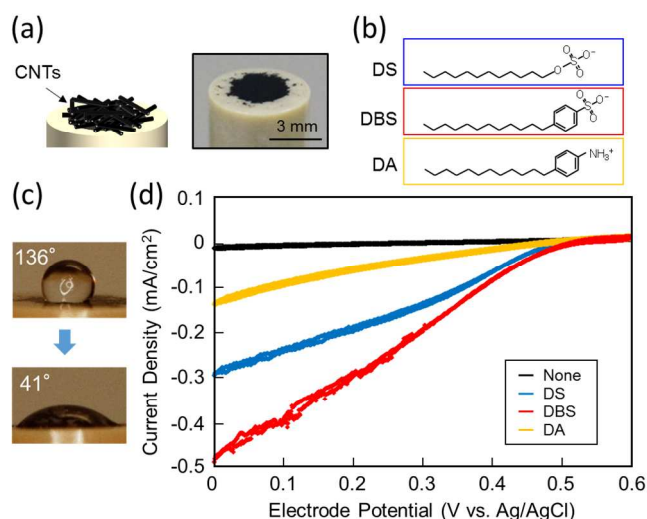
Fig. 1 (a) Schematic illustration and photograph of a random CNT (rCNT) electrode. (b) Molecular structures of surfactants used in this study. (c) Photographs of water droplets placed on rCNT electrodes before and after the surfactant pretreatment. (d) Typical CVs of O_2 reduction at a BOD/rCNT electrode measured at 10 mV s^{-1} in O_2-saturated buffer. The activity of the rCNT electrode was enhanced by surfactant modification with DS (blue), DBS (red) and DA (yellow).

CNTFs were grown on silicon wafers by the methods reported previously.[16,17] The synthesized CNTF films (1 mm × 1 mm), which is composed of aligned 1 mm long single-walled CNTs with a pitch of 16 nm, could be pulled from the substrate with tweezers, as shown in Fig. 2a. The film thickness was set to 12 μm by the width of the line-patterned catalysts used for CNTF growth. The CNTF film electrodes were first treated with 0.1% surfactant solutions. The amount of surfactant adsorbed was evaluated[18] and found to be 0.25~0.45 molecules / nm^2 independent of the kind of surfactant used, as calculated assuming the specific surface area of CNTF is 1300 m^2 / g.[17] This amount of surfactant is one order of magnitude smaller than that of a close-packed monolayer of DBS, 4.3 molecules nm^{-2},[19] indicating the sparse adsorption of DBS (and the other surfactants studied) to the surface of CNTs. For the loading of enzymes, the pre-treated CNTFs were immersed in a stirred

phosphate buffered saline (PBS; pH 7.0) containing 1 mg ml^{-1} BOD for 10 min. The CNTF electrodes were anchored at the edge of the SUS316L fine tweezers that were used for the leads for electrochemical measurements.

Fig. 2b presents the CVs of BOD/CNTF electrodes, showing the presence of surfactants enhances the penetration of enzyme solution and eventually enhances the electrode performance. Fig. 2c shows that the averaged ($n = 4$) current density at 0 V vs. Ag/AgCl depends on the kinds of surfactant used for the pre-treatments. In particular, the treatment with DBS produced a superior effect than DS and DA. It is worthwhile to note that the spread in the data was within 28% due to the well-defined structure of the CNTF. The amount of BOD molecules incorporated inside the CNTF structures were quantified using inductively coupled plasma mass spectrometry (ICP-MS),[20] and were found to roughly correspond to two ~ three lines of BOD (*ca*. 6 nm in diameter) in the interspace of CNTs (Fig. S1, EIS). Importantly, these results indicate that the surfactant-dependent electrode activity does not simply result from the amounts of BOD inside the CNTF. For example, although the DS-modified CNTF contains a comparatively larger amount of BOD, it shows rather smaller catalytic activity than the DBS-modified CNTF. Therefore, it appears that the rate, or probability, of BOD's DET is affected by interactions with the surfactants adsorbed on the CNTs.

The main structural difference between DBS and DA is the charge polarity of the head group; DBS has a negative sulfonate group and DA has a positive amino group. The DET activity of BOD was higher at the negatively charged DBS-modified CNTF than that at the positively charged DA-modified CNTF, in agreement with a previous report that showed smooth Au electrodes modified with self-assembled monolayers of thiols having a negative head group were superior for DET of BOD.[6] Since the local residues near the active sites of BOD have net positive charge at pH 7.0, the negatively charged electrode surface could provide the molecular orientation of BOD suitable for the DET reactions. From a comparison of the effect of DBS and DS, the presence of a benzene ring also seems to be effective in promoting the DET reaction. It is known that the benzene ring of DBS tightly adsorbs to the CNT surface,[21,22] and probably provides a stable interfacial structure for the BOD/surfactant/CNT system. In addition, the π-conjugated benzene ring could serve as an electron pathway for the DET reaction.

Fig. 3 shows the results observed for the LAC-immobilized CNTFs. The loading of LAC into the CNTFs was conducted using a MacIlvain buffer (pH 5.0) containing 0.25 mg ml^{-1} LAC. The effect of the presence of a benzene ring (compare DS and DBS) was
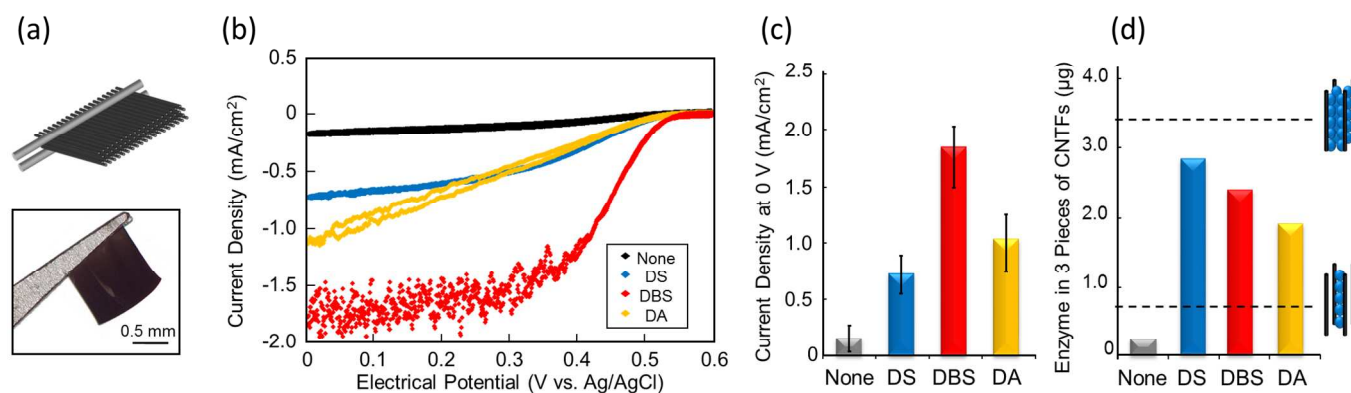
Fig. 2 (a) Schematic illustration and photograph of a CNTF electrode. (b) Typical CVs at 10 mV s^{-1} in O_2-saturated buffer (pH 7.0) for the BOD/CNTF electrodes pre-treated with DS (blue), DBS (red) or DA (yellow). (c) The average current density at 0 V vs Ag/AgCl ($n = 4$). (d) The amount of BOD molecules adsorbed inside CNTF films.

basically the same as for BOD/CNTF electrodes. Unlike the case of BOD, the performance of LAC/CNTF electrode was not critically affected by the charge polarity of the surfactants (compare DBS and DA). Since the isoelectric point of LAC is around 4.0, [8,23] the net charge of LAC becomes close to neutral under the present experimental condition (pH 5.0). This would be a reason of no clear difference between the results obtained at negatively charged DBS-modified CNTF and the positively charged DA-modified CNTFs.
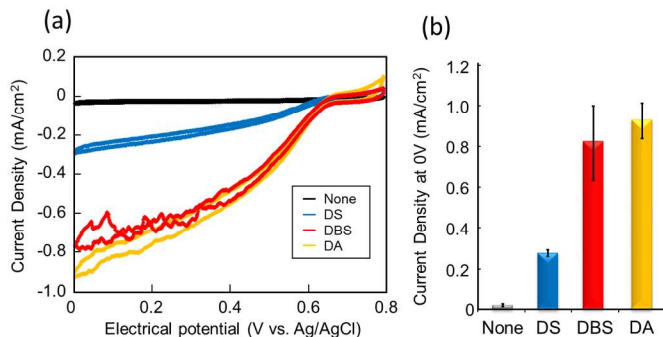
Fig. 3 (a) Typical CVs at 10 mV s^{-1} in O$_2$-saturated buffer (pH 5.0) for the LAC/CNTF electrodes pre-treated with DS (blue), DBS (red) or DA (yellow). (b) The averaged current density at 0 V vs Ag/AgCl ($n = 4$).

In conclusion, we found that pre-treatment of CNT-based nanostructured electrodes with surfactants produces effects not only due to the degree of penetration of the solution into the electrodes, but also on the efficiency of DET reaction of MOC enzymes. The presence of a benzene ring in the molecular structure of the surfactant stabilizes the DET system for O$_2$ reduction. Also the charge polarity of the head group of the surfactants affects the DET activity. In the case of BOD, the negatively charged surfactant (DBS) showed a larger effect on the DET reaction than the positively charged surfactant (DA). These surfactant effects were quantitatively evaluated for a CNT-based 3D nanostructured electrode for the first time by taking advantage of the excellent reproducibility of the well-structured CNTF electrodes.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Methods for calculating the amount of enzymes inside CNTF with supplementary figures. See DOI: 10.1039/c000000x/

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