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Research Article

Enzyme/Carbon Nanotube Aqueous Mixture for Single-Step Printing of Enzyme Electrodes

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Abstract

Advanced biofuel cells consist of nanostructured carbon electrodes modified with enzymes becaise of their higher performance due to larger specific surface area. These nanocarbon-based enzyme electrodes generally require two-step preparation processes, because modification with enzymes is incompatible with harsh conditions for the fabrication of nanostructured electrodes such as heating and use of organic solvent. Here, an aqueous mixture containing enzymes and Carbon Nanotubes (CNTs) is developed by using trehalose as a biocompatible dispersing agent for CNTs. This aqueous mixture can be directly printed on insulating flexible substrates such as textiles. Since the mixture also contains a small amount of binder molecules, the printed electrode maintains structural integrity even in aqueous solution, and it shows catalytic activity in mA/cm^2 level. This single-step printing process enables arbitrary patterning of enzyme electrodes.

Keywords: Printable; Enzyme electrode; Biofuel cell; Carbon nanotube

Introduction

Recent progress in printed electronics opened up the possibilities for thin and flexible electronic devices [1-4]. Thin polymer films are used for these devices to ensure flexibility, and the idea of using a thin flexible substrate is also pursued in the field of paper-based analytical devices that have characteristic advantages such as pump-free sample transport [5-9]. Enzyme-modified electrodes have been used as core components of biofuel cells and biosensors integrated in these devices, where they take advantage of enzymatic reactions to obtain electric power and quantitative information on analytes, respectively [10-27]. The performance of enzyme electrodes has been improved by tailoring composite of enzymes and carbon nanomaterials such as particulate carbon black and Carbon Nanotubes (CNTs). Carbon nanomaterials provide a three-dimensional conductive network and high specific surface area for effective enzyme reactions. For example, CNT-based microfiber [20,21], a film of well-aligned CNTs [22,23], electrospun carbon nanofiber [24], and a nanoporous carbon cryogel [25], have been successfully combined with enzymes to achieve enzyme electrodes with high activity in the range of mA/cm^2. Fabrication of these nanocomposite enzyme electrodes, however, requires two separate steps of preparing a nanostructured electrode and its soaking with enzyme solution for modification. This is because the preparation of nanocarbon electrodes involves conditions that are harsh for enzymes such as heating and treatment with organic solvent or surfactant. Making an enzyme/nanocarbon composite electrode in a single process will ensure more uniform composition even in the interior of the electrode. There have been only a few examples of single-step preparation of enzyme/nanocarbon composite electrodes. Zebda et al. made a composite disk of compressed enzyme/CNT mixture [26,27]. Kwon et al. reported a single-step preparation of CNT yarn with deposited enzymes and mediators from aqueous solution [28]. Recently, Bandodkar et al. succeeded in mixing enzymes with graphite powder-based conductive ink by modifying its composition to be biocompatible [29].

In the present study, we developed an enzyme/CNT composite mixture that can be printed on an insulating flexible substrate such as a nonwoven textile. The aqueous composite mixture was realized by using trehalose as a biocompatible dispersing agent for CNT. The printed enzyme electrode can be preserved in freeze-dried condition, and shows catalytic activity in mA/cm^2 level after rehydration by the aqueous solution of enzyme substrates. This single-step printing process enables arbitrary patterning of enzyme electrodes.

Materials and Methods

Preparation of the Enzyme/CNT composite mixture and printing electrodes

Preparation of the composite mixture starts by dispersing a single-walled super growth CNT (SGCNT; 200 μm in average length) [30,31] or a conventional multi-walled shorter CNT (Bayer, 5 μm length in average) at 1 mg/ml in 25mM McIlvaine buffer (pH 5) containing 10 wt% trehalose and 1 wt% PVI by sonication for 10 min. A 46.5 μl portion of the resulting aqueous CNT dispersion was mixed with 1 μl solution of 1 M PBSE/DMSO to be ca. 20 mM PBSE and placed in iced water for 1 hr. Finally, 2.5μl of 5 mg/ml D-fructose dehydrogenase (FDH; EC 1.1.99.11 from Gluconobacter, Toyobo) was added to the 93μl of the above prepared CNT dispersion. The resulting enzyme/CNT aqueous mixture was printed (40μl / cm^2), frozen in liquid nitrogen, and vacuum-dried at about 10 Pa for 5 h. The amount of trehalose was optimized by measuring the activity of the rehydrated enzyme electrodes, which were freeze-dried with various amount of trehalose. For the preparation of the electrode for glucose oxidation, FDH and PVI were replaced by glucose oxidase (GOD; EC 1.1.3.4, from Aspergillus sp., Toyobo) and polyvinylimidazole-[Os(bipyridine)$_2$Cl] (PVI-Os, MW ca. 15000). The PVI-Os was synthesized according to the literature [32], as the molar ratio of imidazole group to [Os(bpy)$_2$Cl] to be 5.

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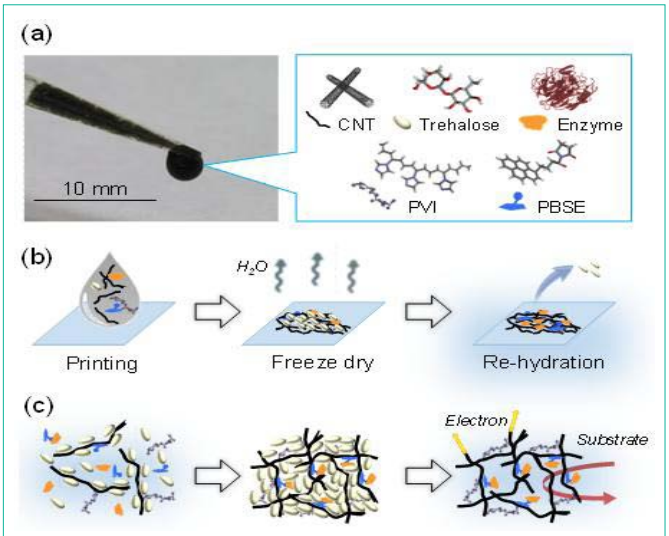


Figure 1: (a) The aqueous mixture composed of CNT, trehalose, enzyme, PVI, and PBSE. (b) The process for preparing enzyme electrodes: printing the mixture, freeze-drying, and rehydration for the use. (c) Schematic illustration of the hypothetical structures of the mixture, the freeze-dried film, and the rehydrated film.

Electrochemical measurements

The CNT/enzyme electrode films were prepared by drawing 40 µl/cm^2 of the composite mixture on a commercial screen carbon electrode (4-mm diameter) or a piece of nonwoven textile. The electrode on the textile was electrically connected to the instruments via SUS316L fine tweezers. Cyclic voltammetry and chronoammperometry were conducted by a three-electrode system (BSA, 730C electrochemical analyzer) in stirred solutions using an Ag/AgCl reference and a Pt counter electrode at room temperature (25 °C).

Biofuel cell experiments

The biofuel cell was constructed by using the printed CNT/FDH electrode on a textile as the anode and a Bilirubin Oxidase (BOD)-modified carbon fabric cathode for O_2 reduction, which was prepared as reported [33,34]. BOD (EC 1.3.3.5, 2.5 U/mg, from Myrothecium) is one of the multi-copper oxidases that can directly catalyze the four-electron reduction of O_2 to H_2O even without electron transfer mediators. The LED device for demonstration of power generation consisted of a charge pump IC (S-882Z20), a 1 µF ceramic capacitor, and a red LED, whose blinking interval is inversely proportional to the power of the biofuel cell [35,36].

Results and Discussion

As shown in Figure 1, the newly developed aqueous mixture is composed of CNT, trehalose, enzyme, and two kinds of binders. A single-walled super-growth CNT (SGCNT, 200 µm average length) [30,31] was typically used. Trehalose was used as an additive for freeze-drying enzyme [37,38], which we also found serves as a dispersing agent for CNTs to give an enzyme/CNT aqueous mixture without surfactant. The printed mixture was immediately freeze-dried for preservation, and rehydrated on its use (Figure 1b). As drawn in the schematic illustration of Figure 1c, the structure and activity of the electrode were expected to be maintained even after rehydration by the effects of two molecular binders in the mixture.

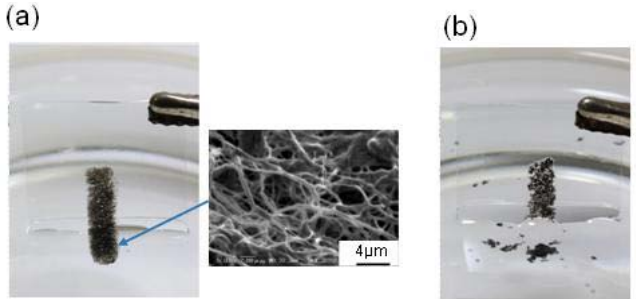


Figure 2: (a) A photograph of the freeze-dried composite film prepared on a slide glass, half of which is dipped into a stirred McIlveine buffer solution. A SEM image shows the microstructure of the rehydrated part of the electrode. (b) A photograph of the film prepared without PVI.

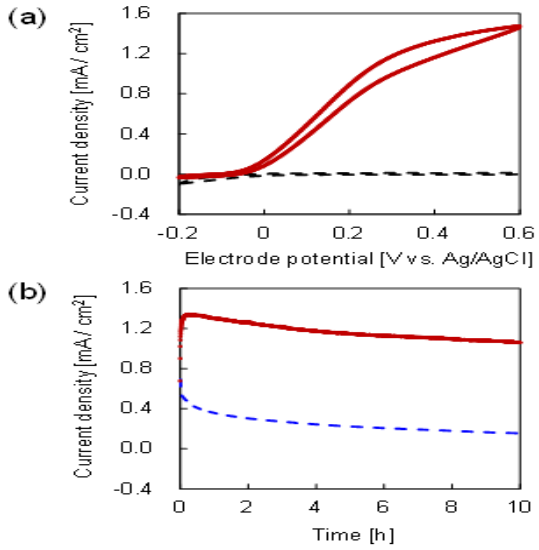


Figure 3: (a) Cyclic voltammogram at 10 mV/s in the stirred McIlvaine buffer (pH 5) containing 0.2 M fructose for an FDH/CNT composite electrode. The film was prepared on a commercial screen carbon electrode (4 mm diameter). The control voltammogram without fluctose is also shown (dotted line). (b) The time course of the current density at 0.5 V vs. Ag/AgCl for the electrode prepared with (red) and without (dotted blue) PBSE.

Polyvinyl Imidazole (PVI) binds CNTs, and 1-Pyrenebutanoic Acid Succinimide Ester (PBSE) does enzymes and CNTs, as proven below.

Figure 2a depicts the freeze-dried composite film prepared on a slide glass, half of which is dipped into a stirred buffer solution. The large amount of trehalose in the composite film is instantly dissolved away into the solution, while the micron-scale network of CNT bundles (SEM image in Figure 2) was left behind. As judged from weighing, more than 85% CNTs remained after rehydration. Importantly, the presence of PVI in the mixture was necessary to maintain the film structure. In fact, the film prepared without PVI immediately collapses on the dipping into the solution, as shown in Figure 2b. The free imidazole groups of PVI could attach to the CNT surface via π–π interaction and bind CNTs together [39]. We observed the similar effect by addition of 0.1 wt% polystyrene sulfonic acid instead of PVI, indicating polymers having aromatic groups could work as a binder.

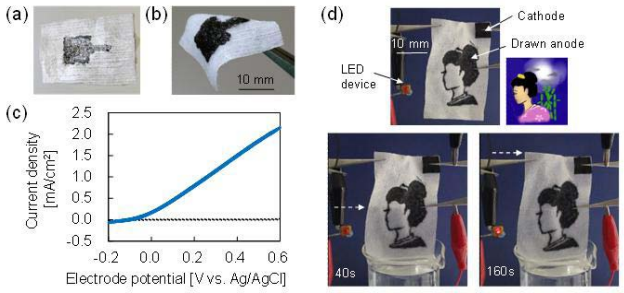


Figure 4: (a, b) Photographs of the drawn FDH/CNT mixture on a nonwoven textile in freeze-dried condition and rehydrated condition. (c) The cyclic voltammogram at 10 mV/s in a stirred McIlvaine buffer (pH 5) containing 0.2 M fructose. (d) Demonstration of a biofuel cell constructed from a drawn FDH anode and the BOD cathode. A pattern of an anode (shadow picture of a lady) was drawn by a stencil mask. A piece of BOD-modified carbon fabric as a cathode was put at the upper right corner of the drawn electrode. The biofuel cell was connected to a LED device and the lower edge of the textile was dipped into a McIlvaine buffer (pH 5) containing 0.2 M fructose. A dashed arrow indicates the moving front of the permeating solution.

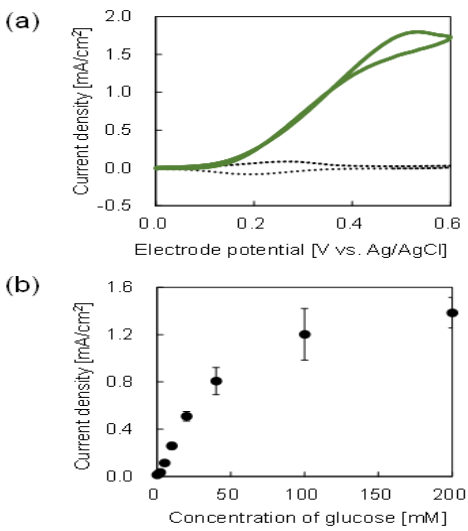


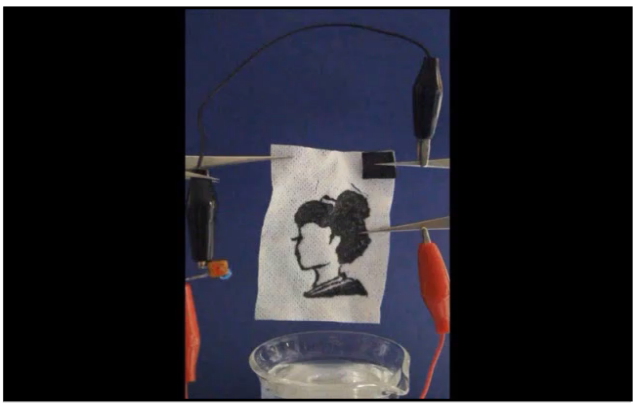
Figure 5: (a) A cyclic voltammogram of the GOD/CNT composite electrode at 10 mV/s in a stirred 50 mM PBS (pH 7) containing 0.2 M glucose. (b) The current densities at 0.6 V as a function of glucose concentration. The mean values (± standard deviation) of three independent specimens are given.

The rehydrated film electrode that is composed of D-Fructose Dehydrogenase (FDH) performed biocatalytic activity (Figure 3). The cyclic voltammogram in a stirred 0.2 M fructose solution showed the maximum current density of 1.3mA/cm^2 at 0.6 V. The electrode performance was reproducible; the maximum current densities of four different electrodes were within the range of 1.1~1.4 mA/cm^2. It should be noted that when the CNT electrode was prepared from CNT dispersion in alcohol prior to enzyme modification (conventional two-step preparation method), the activity of the resulting enzyme electrode was smaller by one order of magnitude (Supplementary Figure S1). The pre-structured dense CNT electrode is unfavourable for both enzyme modification and substrate supply. In contrast, in the case of the present composite electrode, enzymes were uniformly contained inside the electrode, and the micron-scale porous structure of the electrode (SEM image in Figure 2) can serve as the supply route of substrates.

The stability of catalytic current at 0.5 V was monitored in a stirred 0.2 M fructose solution (Figure 3b). During hydration (~ 10 min), the current steeply increased to the maximum, 80% of which was maintained even after continuous monitoring for 10 h. This stability was ensured by PBSE that attaches to the CNT surface and covalently binds to the amino group of FDH [19]. When the electrode film was prepared without addition of PBSE, the electrode activity was lower and soon decreased (dotted blue curve) surely due to leakage of FDH from the electrode film to the solution. This stabilizing effect was also observed by 20 mM N-(benzoyloxy) succinimide instead of PBSE.

The electrode performance was also evaluated for the electrode film prepared using a conventional multi-walled shorter CNT (5 μm length on average) instead of the longer SGCNT (Supplementary Figure S2). The maximum CV current was 0.8mA/cm^2, and 60% of the current was maintained even after continuous 10 h monitoring. It is practically important that even the conventional shorter CNT can be used for the present single-step preparation of enzyme electrodes, while its performance is somewhat lower than that with a SGCNT probably due to the lower conductivity [30].

Printing enzyme electrode is essential for the development of the paper-based and textile-based bio devices. Figure 4a and 4b depicts the FDH electrode printed on a piece of nonwoven textile; (a) freeze-dried condition and (b) rehydrated condition. As shown in Figure 4c, the maximum current density of the electrode on textile was enhanced to 2mA/cm^2 probably due to the larger specific surface area of the textile that is favorable for the electrode modification and the effective diffusion of fructose through the mesh of the textile. These performances were kept even under bending condition, indicating that the FDH/CNT composite was well integrated to each fiber of the textile. It is also important that the printed electrode works even without an underlying conductive substrate, although the linear shape of voltammogram indicates non-negligible resistivity of the printed electrode. Figure 4d shows a complex drawing of the FDH electrode (a shadow picture of a lady) made by the present FDH/CNT mixture with a stencil mask. Importantly, the conventional two-step fabrication of an enzyme electrode is not suitable for preparing the complicated patterns of enzyme electrode. In order to prove that the drawn FDH electrode serves as an anode of a biofuel cell, a piece



Movie S1:

of BOD-modified carbon fabric for O_2 reduction as a cathode was put on the upper right corner of the textile, and these electrodes were electrically connected with metallic tweezers to drive a LED device [35,36]. At 40 s after dipping the lower edge of the textile to an electrolyte solution containing fructose, the moving front of the permeating electrolyte reached the middle part of the textile without significant disruption of the drawn electrode. At 160 s, the electrolyte reached the cathode, and the LED started to blink, demonstrating the drawn electrode served as the anode of a fructose/O_2 biofuel cell (Supplementary Movie S1).

The composite electrode for oxidation of glucose was prepared using D-Glucose Oxidase (GOD). Since the electro catalytic reaction of GOD requires a suitable mediator, the Os complex-modified PVI (PVI-Os) [23] was added to the printable enzyme mixture instead of the native PVI. As shown in Figure 5a, the oxidation current more than 1.5mA/cm^2 was observed in the presence of 0.2 M glucose. The oxidation currents were dependent on the concentration of glucose (Figure 5b), indicating possible application to glucose sensing [10,40]. These results prove the versatility of the present single-step printing process for preparing enzyme electrodes regardless of the kind of an enzyme.

Conclusion

In conclusion, we have developed an aqueous enzyme/CNT mixture by using trehalose as a biocompatible dispersing agent for CNTs, which enables versatile single-step printing of an enzyme electrode on any substrates including flexible textile. Due to the effects of binder molecules, PVI for CNT / CNT and PBSE for enzyme/CNT, the printed electrode maintains its structure even in aqueous solution, and shows catalytic activity in mA/cm^2 level. Applications to a biofuel cell on a nonwoven textile were demonstrated. This single-step printing process enables arbitrary patterning of enzyme electrodes, such as a complex drawing presented here, and will contribute to the widespread use of enzyme electrodes that are integrated into flexible devices.

Acknowledgement

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