# Powerful connection of laccase and carbon nanotubes. Material for mediator-free electron transport on the enzymatic cathode of the biobattery

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## Abstract

We describe the preparation of laccase/single-wall carbon nanotube bioconjugates, their application for the modification of electrodes and application of the electrodes as cathodes for the catalytic reduction of oxygen in a hybrid biofuel cell with Zn anode. Carbon nanotubes functionalized with aminoethyl residues, activated and reacted with laccase show high bioelectrocatalytic activity and are promising for the biofuel cell applications. The power density of the cell was 1 mW cm$^{-2}$ at working voltage of 0.8 V. The open circuit voltage of this hybrid cell was as high as 1.5 V.

**Key words:** direct electron transfer, laccase, oxygen reduction, biofuel cell, carbon nanotubes, bioelectrode.

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## 1. Introduction

Enzymatic biofuel cells have attracted considerable attention as alternative, cheap and environmentally friendly power sources that also could be implanted into living body. [1-2] They utilize some small compounds (e.g. glucose) present in body as natural fuel, and convert chemical energy into electrical power with the use of redox enzyme as catalyst under ambient conditions and in physiological pH. To facilitate electron transfer between enzyme and electrode various mediators are usually employed. [3-6] Methods based on direct electron transfer would, however, be cheaper and much more convenient in practical applications. Electrodes nanostructured with carbonaceous materials show increased efficiency of the non-mediated bioelectrocatalytic processes. Polytetrafluoroethylene-Ketjen black composite deposited on carbon paper was used as the support material for copper efflux oxidase in contact with air exhibited an extremely high catalytic current density of ca. 20 mA $cm^{-2}$. [7] Hydrophobic carbon black was employed for the construction of a “floating” air diffusion biocathode with Trametes Hirsuta laccase as catalyst gave current densities of 0.5 mA $cm^{-2}$. [8] Gas-diffusion electrode based on carbon black hydrophobized with Teflon with immobilized laccase exhibited catalytic current density of 1 mA $cm^{-2}$. [9] Carbon nanotubes are used in bioelectrodes to improve conductivity and to increase the working electrode surface and the amount of enzyme directly connected to the electrode surface. [10-13] There are only few efficient direct electron transfer-type (DET-type) biocathodes designed for a biofuel cell, and even less - with the enzyme covalently bound to a nanostructured electrode surface as those constructed in our work. [14,15, 16, 17] The main goal of our work was to construct an efficient biocathode for the reduction of dioxygen to water based on direct electron transfer in the laccase – carbon nanotube conjugate network. Such approach eliminates the use of mediators together with increasing the number of laccase molecules in favorable orientation for electron exchange with the electrode. Important for the biofuel cell applications high catalytic current and good stability of the biocathode can be achieved. As shown earlier, direct laccase adsorption on other carbonaceous materials, e.g. pyrolytic graphite suffers from poor stability. Surface modification with anthracene on which laccase molecule can be anchored improves both stability and catalytic activity. However the drawback of this solution is that the single anthracene layer is not able to fix many laccase molecules. [18,19]

In the present work, single wall carbon nanotubes (SWCNT) were functionalized (on walls or at ends) with linkers of different length terminated with amino groups [Scheme 1- A]. The SWCNT with amino groups were treated with glutaraldehyde (GA) [Scheme 1-B] and then reacted with polymerized or non-polymerized laccase. Treating enzyme with GA produced material called the polymeric laccase. Glassy carbon electrodes (GCE) covered with laccase-SWCNT conjugates were sufficiently stable and useful for application in the hybrid biofuel cell.

# 2. Material and methods

## 2.1 Materials and equipment

Laccase from *Cerrena unicolor* C-139 was obtained from the culture collection of the Regensburg University and deposited in the fungal collection of the Department of Biochemistry (Maria Curie-Sklodowska University, Poland) under the strain number 139. Laccase from the fermentor scale cultivation was obtained according to already reported procedure. [6, 10] After lyophilising, the laccase activity dissolved in 1 ml of water was 92,000,000 nkat $dm^{-3}$ and $C_{lacc}$ = 15 mg $cm^{-3}$.

Carbon nanotubes (>90%) were purchased from CheapTubes.com. and characterized in [10, 11].

$Na_2HPO_4$, $KH_2PO_4$ and citric acid were from Polish Chemicals Co. All solutions were prepared using Milli-Q water (18.2 MΩ $cm^{-1}$), Millipore, Bedford, MA, USA. Cyclic voltammetry experiments were performed using a CHI 700B bipotentiostat in a three-electrode arrangement with a silver-silver chloride reference electrode (Ag/AgCl) and a platinum sheet as a counter electrode. The film laccase-SWCNT conjugate modified GCE was the working electrode. All current densities were calculated using geometrical area of the GCE (BAS) (A=0.071 $cm^2$). The biofuel cell parameters were examined in dioxygen saturated 0.15 M McIlvaine buffer solution, pH 5.3. Open circuit voltage (OCV) was measured in all experiments. The cell current ($I_{cell}$) and the cell voltage ($V_{cell}$) were measured under varying loading in the range from 1 kΩ to 10 MΩ; current and cell voltage were measured after stabilization of the system achieved 30 s after each load application.

## 2.2 Preparation of laccase-SWCNT bioconjugates

Here Scheme 1

Oxidized nanotubes were converted into acid chlorides, reacted with ethylenediamine giving **SWCNT-CO-NH-$(CH_2)_2$-$NH_2$ (1)** [20,21] or with cold aqueous ammonia, giving **SWCNT-CO-$NH_2$ (M-2)** [Scheme 1-A]. The nanotubes modified with aminoethyl groups on the side-walls **SWCNT-$(CH_2)_2$-$NH_2$ (3)** were synthesized as described earlier. [23]

SWCNT derivatives (**1**, **2**, **3**) were used for covalent laccase immobilization [Scheme1-B]. Two strategies of laccase immobilization were applied.

Procedure 1: Materials **1**, **2** and **3** were treated in Eppendorf tube with glutaraldehyde (5% in 0.1 M phosphoric buffer pH 7.0) according to [24], sonicated for 4 h and then rotated for 8 h in Rotator (Neolab, Heidelberg, Germany) at 10 rpm. The samples were centrifuged at 10 000 x g for 8 min, three times washed with water and centrifuged as above. Next the laccase solution was added to suspension of modified carbon nanotubes and allowed to react for 12 h at 10 rpm rotation and washed as above.

Procedure 2: Primary amino groups of laccase were first activated and cross-linked with glutaraldehyde (5% in 0.1M phosphoric buffer pH 7.0) for 8 h at 20°C (10 rpm rotation). The reaction mixture was next purified by removal of not reacted glutaraldehyde with protein by ultrafiltration on Amicon Ultra-2 filter (10 kDa cut off membrane) using 10 000 x g force. The concentrate was diluted to the original volume and centrifuged at 10 000 x g force. Triple repetition of the procedure removes free GA from the polymeric laccase solution. Such polymerized laccase solution was then mixed with activated by GA materials 1, 2 or 3 as in procedure 1.

### 2.3 Electrode modification procedures

GCE electrode was polished with aluminum oxide powder (grain size down to 0.05 µm) on a wet pad, rinsed with water and ethanol, dried at room temperature and used for modification. Suspension of laccase-functionalized SWCNT in water was dropped onto the electrode surface. Electrodes were left to dry under the argon stream and then used in experiments. The anodes for the Zn-$O_2$ hybrid fuel cells were zinc wires (0.25 mm diameter, Goodfellow) coated with Nafion by dipping in 0.5% Nafion solution in methanol and drying for 5 minutes in ambient conditions. A hopeite layer formed during Zn electrode oxidation prevents Zn corrosion, as described in literature. [25, 26] The length of these electrodes was adjusted to obtain the same surface area as that of the biocathode. Cathodes based on laccase-SWCNT bioconjugates were tested in Zn/$O_2$ hybrid fuel cell. Loads, in the range from 1 kΩ to 10 MΩ, were applied between the Zn/Nafion anode and the cathode to determine the cell current ($I_{cell}$) and the cell voltage ($V_{cell}$).

## 3. Results and Discussion

Electrodes modified with each of the laccase-SWCNT conjugates were used for dioxygen reduction. On the glassy carbon electrode covered with conjugate **1a** [Scheme 1-B] in the presence of dioxygen, the catalytic reduction current was observed. Dioxygen reduction starts at ca. 600 mV *vs.* Ag/AgCl which is close to the potential of T1 site of laccase *Cerrena unicolor* C-139. After 3 h of continuous work, the current density of dioxygen reduction decreased by ca. 15%. If conjugate **2a** was employed only negligible catalytic current was detected. It seems that linker in conjugate **2a** is probably too short and does not allow for appropriate electrical connection of laccase with the electrode although as checked and should be underlined - laccase is present on the electrode and remains active in solution activity tests. Dioxygen reduction proceeds at T1 site of laccase which is located in the hydrophobic pocket of enzyme, far from the electrode surface and electron transfer between enzyme and the electrode is not favored using this linker. Observations made in affinity chromatography suggest that the ligand should be separated from the matrix at least by 6 carbons in order to be recognized by the enzyme.

Here Fig. 1

Figure 1A presents cyclic voltammograms recorded on glassy carbon electrodes modified with conjugate **3a**. In the presence of dioxygen well developed catalytic curve can be observed, with the current density of 0.6 mA cm$^{-2}$. Adding fluoride ions to solution results in the disappearance of the catalytic current confirming catalytic activity of laccase conjugated with these SWCNT. Fluorides are strong inhibitors of laccase, they bind to T2/T3 cluster of laccase blocking the dioxygen reduction process. The laccase-SWCNTs bioconjugates exhibit high stability and remain active after several months. Procedure 2 was carried out in order to increase laccase quantity on the electrode surface and to improve durability of the biocathode. [Fig. 1B] Mechanical stability and durability was improved. Within 3 h of continuous working no changes of voltammetric response were observed. On the other hand when conjugates **1b**, **2b** and **3b** were used no increase of catalytic current was observed as compared to conjugates **1a**, **2a** and **3a**. This would mean that only laccase molecules directly connected to the carbon nanotubes contribute to the catalytic reduction of dioxygen at the electrodes.

Power and voltage dependences on the current density for the cells consisting of A) **GCE/1a**; B) **GCE/3b** biocathodes and Zn anodes are shown in Fig. 2.

Here Fig. 2

The open circuit potential (OCP) measured for all systems was ca. 1.5 V which is close to the difference in the formal potentials of the half cell reactions (ca. 1.5 V). The cell performance was examined under the varying loads. For the system based on **GCE/1a** the power density was 0.2±0.03 mW $cm^{-2}$ at 0.3 V, whereas 0.5±0.06 mW $cm^{-2}$ was achieved with **GCE/3b**, in case of which the nanotube functional groups did not contain oxygen. In case of **GCE/3a** the measured power density was about 1±0.1 mW $cm^{-2}$. Four cells combined together switched on the light-emitting diode (3V).

## 4. Conclusions

We present a simple construction of biocathode based on non-mediated electron transfer between laccase and the electrode. Laccase is bound covalently to functionalized carbon nanotubes and no mediators are needed to exchange electrons with the electrode. The best electrodes were obtained with conjugates based on **SWCNT-$(CH_2)_2$-$NH_2$ (3)**. Implementation of conjugates with polymerized laccase (**3a** and **3b**) results in better durability of the modified electrode. The power density of a hybrid biofuel cell consisting of $Zn/Zn^{2+}$/Nafion anode and nanotube modified **GCE/3a** cathode is ca. 1 mW $cm^{-2}$ at 0.8V, and in case of **GCE/3b** cathode is 0.5 mW $cm^{-2}$ at 0.6V.

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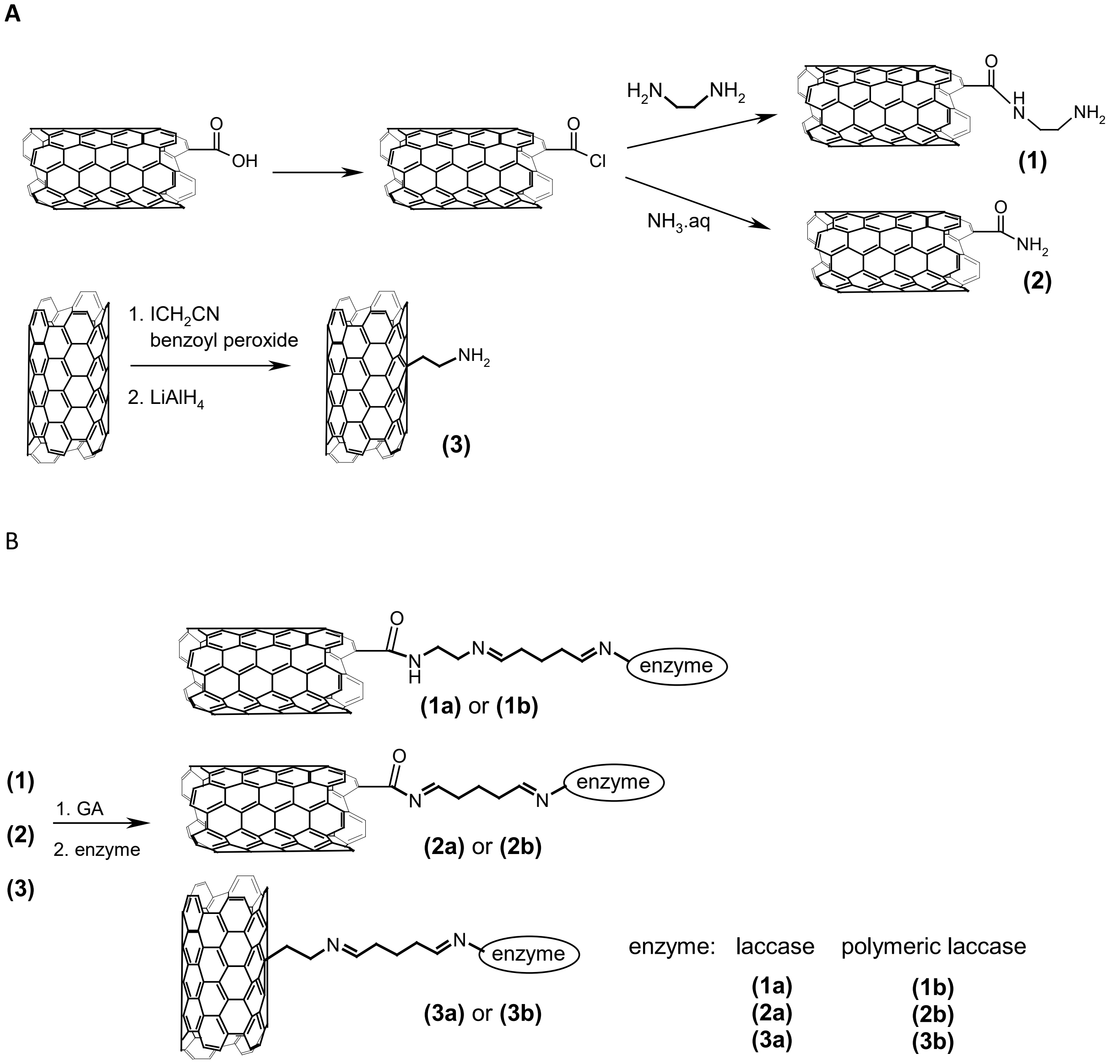
Figure captions

Scheme 1 A) Schematic representation of functionalized carbon nanotubes: (**1**) SWCNT-CO-NH-$(CH_2)_2$-$NH_2$), (**2**) SWCNT-CO-$NH_2$ and (**3)** SWCNT-$(CH_2)_2$-$NH_2$), B – Scheme of laccase conjugation with carbon nanotubes.

Fig. 1 Dioxygen reduction on electrode modified by dropping laccase suspension (see Materials and Methods) A) 30 µl **3a**, B) 10 µl **3b** onto the electrode surface and dried. Scan rate 1 mV/s.

Fig. 2 Dependence of the power density and voltage on the current density of the hybrid fuel cell. Cathode: A**) GCE/1a**, B) **GCE/3b**; Anode: Zn/Nafion.

## Scheme 1


A
$H_2N$ $NH_2$
$NH_3$.aq
O
OH
O
Cl
O
H
N
$NH_2$
(1)
O
$NH_2$
(2)
1. $ICH_2CN$
benzoyl peroxide
2. $LiAlH_4$
$NH_2$
(3)
B
(1)
(2)
(3)
1. GA
2. enzyme
O
N
H
N
N
enzyme
(1a) or (1b)
O
N
N
enzyme
(2a) or (2b)
N
N
enzyme
(3a) or (3b)
enzyme:
laccase
polymeric laccase
(1a)
(2a)
(3a)
(1b)
(2b)
(3b)

Figures

Figure 1

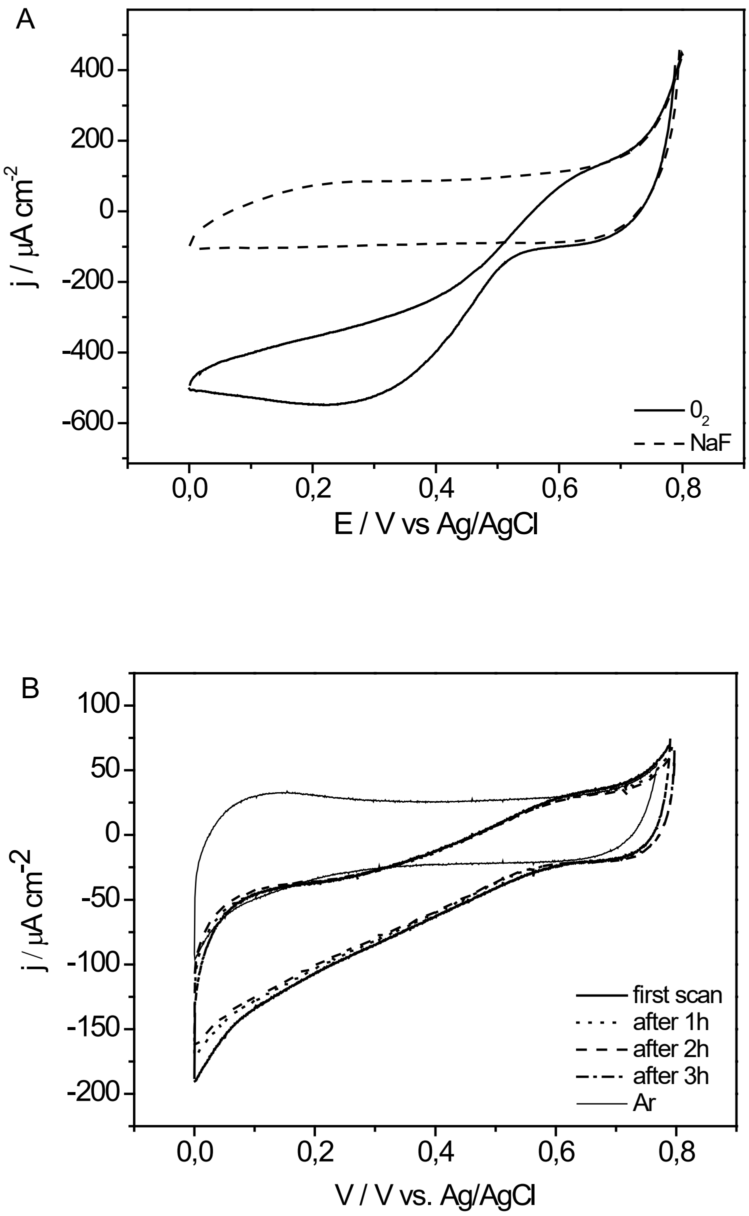
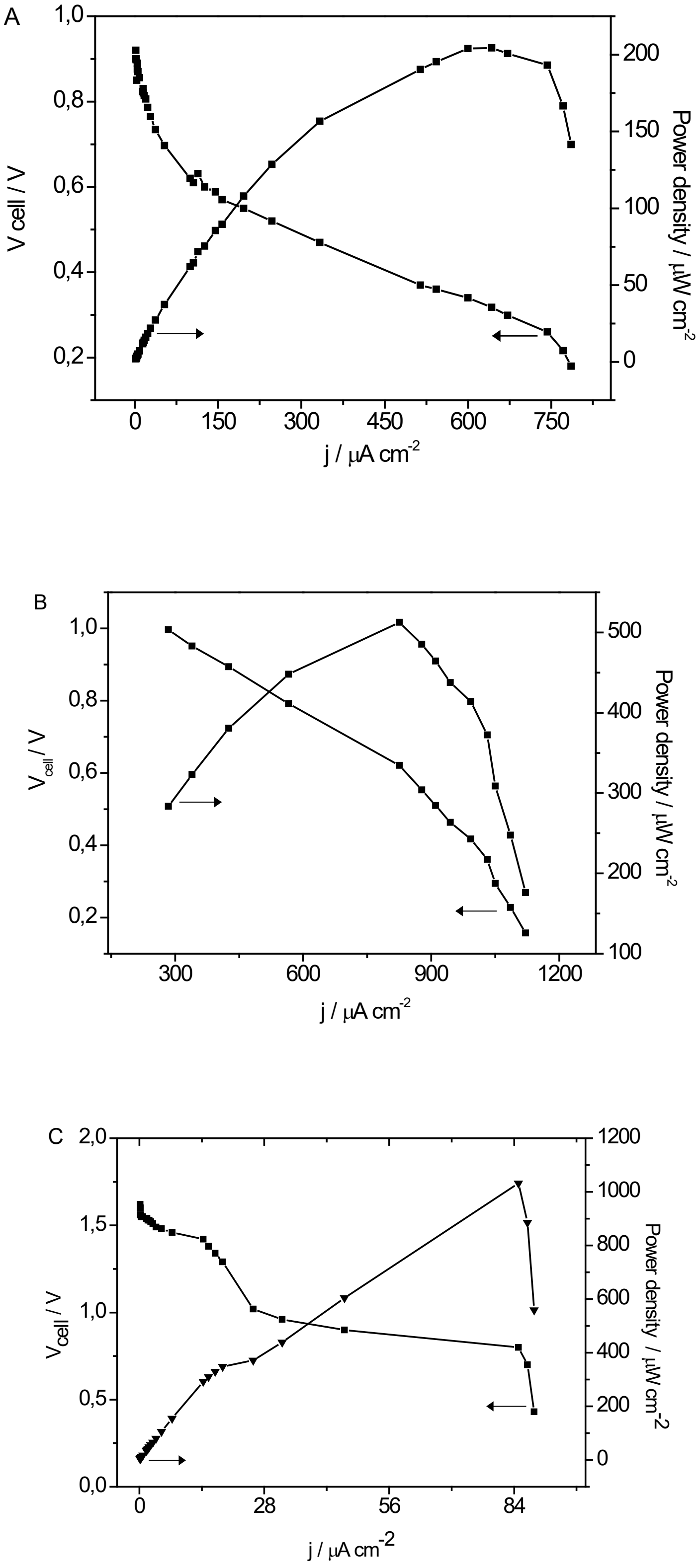


Figure 2


A
V cell / V
1,0
0,8
0,6
0,4
0,2
Power density / µW cm$^{-2}$
200
150
100
50
0
0
150
300
450
600
750
j / µA cm$^{-2}$
B
V$_{cell}$ / V
1,0
0,8
0,6
0,4
0,2
Power density / µW cm$^{-2}$
500
400
300
200
100
300
600
900
1200
j / µA cm$^{-2}$
C
V$_{cell}$ / V
2,0
1,5
1,0
0,5
0,0
Power density / µW cm$^{-2}$
1200
1000
800
600
400
200
0
0
28
56
84
j / µA cm$^{-2}$

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