



Short communication

Bienzyme reactions on cross-linked DNA scaffolds for electrochemical analysis



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ABSTRACT

Enzymes play an essential role in various detection technologies. We show here that interstrand cross-linked oligodeoxynucleotides (CL-ODNs) can provide stable scaffolds for efficiently coupling two types of enzymatic reactions on an electrode. Glucose can be electrochemically detected using glucose oxidase (GOx) and horseradish peroxidase (HRP). When both GOx and HRP were immobilized on an electrode surface by attachment at the termini of CL-ODNs, the current value was markedly increased compared with that obtained on a standard ODN scaffold. The relative orientation of the enzymes on the electrode strongly affected the current intensities. The CL-ODN also allowed GOx–HRP to form a complex on the tiny surface of a microelectrode, resulting in the imaging of local glucose distribution. These results suggest that CL-ODNs have potential utility in other sensing technologies.

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1. Introduction

Glucose oxidase (GOx) plays an essential role in glucose-sensing technologies [1,2]. GOx catalyzes the aerobic oxidation of β -D-glucose to gluconolactone with the simultaneous production of hydrogen peroxide. Glucose concentrations can be determined with excellent sensitivity using a coupled reaction system involving GOx and other enzymes. When GOx is immobilized with horseradish peroxidase (HRP) on an electrode surface, hydrogen peroxide is rapidly reduced by HRP. The oxidized form of HRP can then be reactivated through reduction by an electron mediator under low applied potentials (Schematic 1A) [3–5] or through direct electron transfer [6–8]. These sequential reactions not only allow the sensitive detection of glucose but are also applicable at the cathodes of biofuel cells [9]. In these bienzyme reactions, the distances between the enzymes as well as their relative positions are important factors. Covalent conjugation [10], avidin–biotin bridges [11,12], polyelectrolyte microcapsules [13], and layer-by-layer polymer films [14,15] have been used in an attempt to regulate the distances between the enzymes or their relative positions.

Double-stranded oligodeoxynucleotides (ODNs) can offer nanosized platforms for DNA hybridization assays [16] or enzyme reactions [17–19]. Therefore, oxidation of glucose has been performed by attaching GOx to standard ODN duplexes immobilized on a disc-type electrode [20,21]. Muller and Niemeyer attached both GOx and HRP to termini of double-stranded ODNs and succeeded in detecting glucose using a colorimetric assay [22]. Although synthetic ODNs provide scaffolds for

enzyme reactions, standard ODN duplexes are always associated with a risk of dissociation into single strands by the influence of environmental physicochemical factors. This instability in ODN scaffolds directly decreases the amount of enzyme present on a solid surface, resulting in low detection sensitivities. Elongation of ODNs overcomes the instability but is not suitable for controlling the distance between enzymes. Previously, we developed a method for interstrand cross-linking (CL) that stabilizes double strands by forming a covalent bond between a pair of apurinic/apyrimidinic sites (AP sites) [23]. In addition, a CL-ODN duplex is resistant to bending because of its rigid conformation [24]. Therefore, we hypothesized that CL-ODN duplexes could offer stable scaffolds for enzyme complexes to catalyze a sequential reaction. We have now constructed electrodes that have both GOx and HRP on various ODN duplexes to develop an optimum ODN scaffold. We describe here the potential of these CL-ODNs and the importance of the orientation of these enzymes on the ODN scaffolds in the electrochemical detection of glucose.

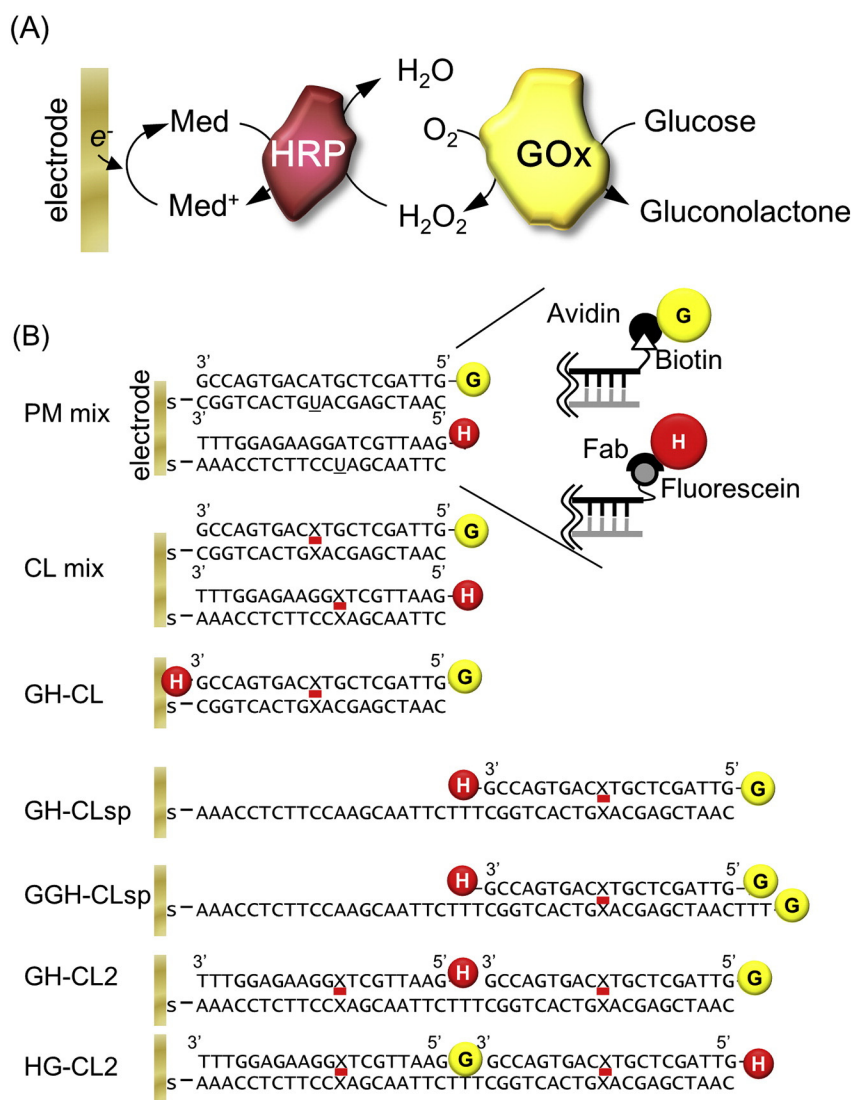
2. Experimental

2.1. Preparation of oligodeoxynucleotides

All oligodeoxynucleotides (ODNs) were chemically synthesized using standard phosphoramidite chemistry at a 0.2 or 1.0 μ mol scale. Amidite units were purchased from Glen Research (USA). The synthesis was conducted with an Applied Biosystems DNA/RNA synthesizer (model 3400). ODNs were purified as previously reported [25]. To immobilize ODNs on a gold electrode, 5'-thiol groups were introduced using dithiol phosphoramidite (DTPA, Glen Research) as previously

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Schematic 1. (A) Schematic of electrochemical glucose detection by a coupled reaction using GOx and HRP. (B) ODNs immobilized on each electrode. U, deoxyuridine; s, dithiol linker; b, biotin; f, fluorescein; X, cross-linked abasic site; bold red lines, cross-linker; G, GOx; H, HRP.

reported [26]. 5'-Dithiol and 3'-biotin-modified ODNs were synthesized using 3'-biotin TEG CPG (Glen Research).

3'-Fluorescein-labeled bf-I (Fig. S1 in SI) was prepared by the postsynthetic modification of 3'-amino- and 5'-biotin-labeled ODN (b-I-NH₂). Here b-I-NH₂ was synthesized using a 5'-biotin amidite unit (Glen Research) and the 3'-PT-amino-modifier C6 CPG (Glen Research). After the standard purification steps, b-I-NH₂ (6 nmol) was labeled with FITC in a solution containing 10% *N,N*-dimethylformamide, 250 mM bicarbonate buffer, and FITC (400 nmol) at room temperature. 3'-Fluorescein-labeled bf-I was purified with HPLC using a reverse-phase column. All interstrand cross-link (CL) duplexes (Fig. S1 in SI) were prepared as previously reported [23].

2.2. Preparation of gold electrode immobilizing CL duplexes and enzymes

Glucose oxidase avidin (GOx-Av) and the horseradish peroxidase anti-fluorescein Fab fragment (HRP-Fab) were purchased from Rockland (USA). Dulbecco's phosphate-buffered saline (PBS) and ferrocenemethanol (FeOH) were purchased from Sigma-Aldrich (USA). All other reagents were analytical reagent grade, and solutions were prepared with ultrapure water obtained using a Millipore system.

Au disc electrodes ($\phi = 3$ mm, ALS Co., Ltd., Japan) were prepared as described in a previous report [27], and ODNs (0.2 μ M) dissolved in a

0.5 M phosphate buffer (pH 7) were dropped onto the electrode (9 μ L). After the gold electrode was incubated in a humid chamber at room temperature for 16 h, it was washed in water (30 mL) at 30 °C for 10 min with moderate stirring. Masking of the surface was conducted by immersing the electrode in 1 mM mercaptohexanol aqueous solution for 60 min, and the electrode was washed twice with PBS solution. The electrode was further masked with BSA solution (1% BSA, 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl) for 2 h to reduce nonspecific binding of proteins and then washed with PBS solution. To bind GOx-Av and HRP-Fab with biotin- and fluorescein-modified ODNs, the gold electrode surface was covered with a GOx-Av (0.01 mg/mL) and HRP-Fab (0.1 mg/mL) solution (9 μ L) containing 1% BSA, 0.15 M NaCl, and 0.1 M Tris-HCl buffer (pH 7.5) and incubated for 60 min at room temperature in a humidified chamber. The electrode was washed three times in PBS solution (30 mL) with moderate stirring. The enzyme-immobilization electrodes were stored at 4 °C in PBS when not in use.

2.3. Monitoring of current response at the GOx and HRP co-immobilization electrode

Batch electrochemical measurements were carried out in a conventional one-compartment cell with a three-electrode configuration using a Hokuto Denko HA1010mM2B potentiostat/galvanostat (Hokuto

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