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Electrochemistry and electrocatalysis of covalent hemin-G4 complexes on gold

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ABSTRACT

Peroxidase-mimicking catalytic DNAzyme labels comprising hemin-guanine quadruplex (G4) complexes are widely used in electrochemical DNA and aptamer assays. However, their catalytic activity and thus the assay performance may be limited by the complex stability. Here, electrochemical properties of the hemin-G4 complexes with hemin covalently attached to the G4 sequence (covalent complexes) and of the non-covalent hemin-G4 complexes were studied and compared in the reaction of electrocatalytic reduction of H_2O_2. At pH 7, non-covalently and covalently bound hemin-G4 complexes immobilized on gold electrodes showed formal potentials of − 242 and − 220 mV vs. Ag/AgCl, being less negative than − 316 mV exhibited by hemin attached to the alkanethiol SAM. Both complexes were able to electrocatalytically reduce H_2O_2 starting from 0.4 V, and O_2 – from 0.15 V. In the reaction of H_2O_2 reduction at 0.2 V, within the potential window of no interference from O_2, the covalent hemin-G4 complex showed a 20 fold higher specific electrocatalytic activity compared to the non-covalent analogue and the K_M (towards H_2O_2) of 0.58 mM (2 mM for the non-covalent complex). Both the enhanced catalytic activity and sensitivity for H_2O_2 were ascribed to the higher integrity/stability of the covalent complexes. The results open new biosensor perspectives for the covalent hemin-G4 complexes as more stable and active labels for DNA and aptamer assays.

1. Introduction

Nucleic acid (NA)-based catalytic analogues of enzymes, so called DNAzymes, are now widely used to catalyse such reactions as DNA/RNA cleavage [1], a number of DNA modifications [2] and porphyrin ring metalation [3]. Compared to the natural enzymes, the DNAzymes are synthetically available and offer such advantages as pH insensitivity and thermostability of their structures [4].

A hemin-guanine quadruplex (G4) complex is one of the most technologically attractive DNAzymes finding broad applications as a catalytic label in DNA biosensors [5]. It is a peroxidase-mimicking DNAzyme, and similar DNAzymes can be produced by complex formation between G4-containing sequences and any other metal containing porphyrins and phthalocyanines [6]. In such complexes, the catalytic activity of free ligands is enhanced by stacking or embedding them into G-rich sequences, and in optical assays, the formed DNAzymes can efficiently catalyse H_2O_2-mediated oxidation of different substrates [5].

That is particularly true for the hemin-G4 complexes [5], which formation significantly enhances the catalytic activity of free hemin in the reaction of H_2O_2 reduction [7]. This property was successfully used in chemiluminescence [8], colorimetric [9] and surface plasmon resonance [10] biosensors for, among other, sensitive analysis of DNA, adenosine monophosphate, and inorganic ions [11]. In all these assays, hemin necessary for the hemin-G4 complex formation was present in solution, and the catalytic properties of the free form did not interfere with the assay performance, though, the sensitivity of the assays is expected to be worse than shown with peroxidases: the limit of chemiluminescent detection of horseradish peroxidase (HRP) is 0.25 pM, and only 350 pM DNAzyme produce a detectable chemiluminescent signal [12].

In electrochemical assays, hemin present in solution may not only result in a worse sensitivity of analysis, but also interfere with the assay results/produce artefacts, since both free hemin and hemin adsorbed on electrodes can exhibit essential electrocatalytic activity in the H_2O_2 reduction reaction [13]. The straightforward strategy is then to reduce the concentration of hemin in solution to the level insignificant for direct electrochemical/electrocatalytic reactions. However, as has been shown, decreasing the hemin concentration to 1 μM (the K_d levels) results in destabilization of the DNAzyme and a partial dissociation of

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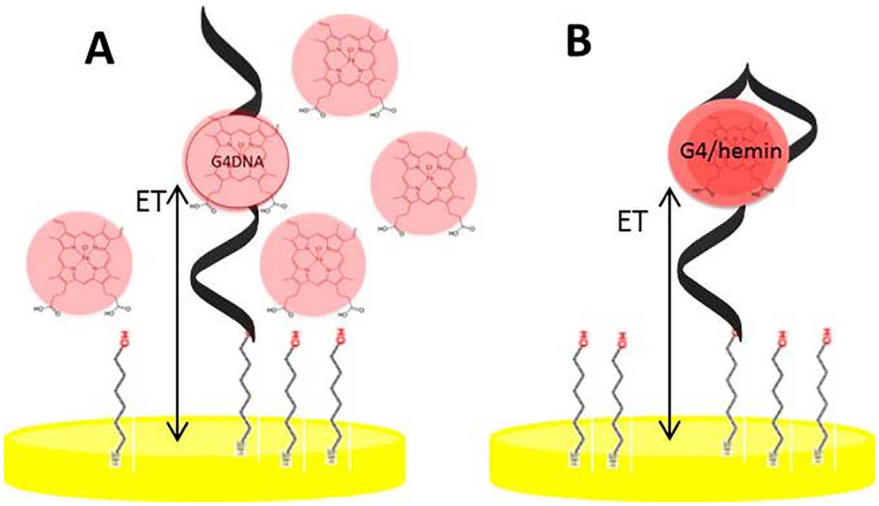

Fig. 1. Schematic representation of (A) a non-covalently formed hemin-G4 complex, with hemin in solution, and (B) a covalent hemin-G4 complex. Both complexes are attached to the gold electrodes via the C_6 alkanethiol linker, with the electrode surface additionally blocked with mercaptohexanol.

hemin from the DNAzyme complex [12]. That is considered as one of the reasons of a lower sensitivity of DNAzyme assays [12]. It was suggested that for improvement of the DNAzyme stability and conservation of its integrity, hemin should be covalently attached to G4 via a flexible T_x "arm" sequence, in order to have a conformational freedom to freely interact with G4. The lowest dissociation constants, therewith, were shown for the covalent hemin-G4 complexes in which hemin was attached through the T_{10} spacer at a 5′-end of a DNA sequence: such complexes demonstrated higher activities in a chemiluminescent assay compared to complexes formed by hemin covalently attached either to the 3′-end of the G4-containing DNA sequence or through the shorter length spacers [6].

In the current work, electrochemical reactivity of the covalent and non-covalent hemin-G4 complexes in the reaction of H_2O_2 reduction was interrogated and compared (Fig. 1), in order to find the best candidate for DNA-based electrochemical assays in which this DNAzyme complex can be used as a peroxidase-mimicking label [14].

2. Materials and methods

2.1. Materials

Hemin, ethyl carbodiimide hydrochloride (EDC), sulfo-*N*-hydroxysuccinimide (NHS), hydrogen peroxide, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), mercaptohexanol (MC_6OH), amino-hexanethiol (MC_6NH_2), NaOH, dimethylsulfoxide (DMSO), ethanol and all buffer components were from Sigma-Aldrich (Broendby, Denmark). The G4-DNA sequences (5′-TTT TTT TTT T CTG GGA GGG AGG GAG GGA TTT TTT TTT T-C6-SH and 5′-NH_2-C_6-TTT TTT TTT T CTG GGA GGG AGG GAG GGA TTT TTT TTT T-C6-SH, both referred further in the text as G4 and amine-G4) and arbitrary DNA, T_{25} (5′-TTT TTT TTT TTT TTT TTT TTT TTT T-C6-S-S-C6-OH) were from Metabion (Planegg, Germany).

2.2. Covalent coupling of hemin to G4

5 μL of a 5 μM amine-G4 solution in a 20 mM phosphate buffer solution, containing 0.15 M NaCl, pH 7.0 (PBS) were mixed with hemin, sulfo-NHS and EDC, to provide their final concentrations of 1, 100 and 5 mM, respectively. The mixture was incubated for 3 h at rt for covalent coupling between the −COOH group of hemin and the $-NH_2$ group of amine-G4. Then, the mixture was dialyzed for at least 1 h at rt (1 kDa cut-off membrane) to remove excess of hemin.

2.3. Electrode modification with G4 and a covalent hemin-G4 complex

Prior modification, gold electrodes (CH Instruments, Austin, TX; 2 mm in diameter) were cleaned by potential cycling in 0.5 M NaOH (from 0.4 to − 1.6 V, scan rate 0.1 V s^{-1}, 10 cycles) and washed with water. Then the electrodes were successively mechanically polished in 1 μm diamond and 0.1 μm alumina slurries (both from Struers, Copenhagen, Denmark). After mechanical polishing, the electrodes were ultrasonicated for 5 min in a 1:1 water-ethanol mixture and electrochemically polished in 1 M H_2SO_4 (from − 0.3 to 1.7 V, scan rate 0.3 V s^{-1}, 10 cycles) and 1 M H_2SO_4 containing 1 mM KCl (from 0 to 1.7 V, 0.3 V s^{-1}, 10 cycles), respectively. The electrochemically active surface area of the electrodes determined from the gold oxide reduction peak in 0.1 M H_2SO_4 was 0.087 ± 0.005 cm^{-1} (the surface roughness around 2.5). The clean electrodes were rinsed thoroughly with Mili-Q water and kept in absolute ethanol for 30 min before modification.

2.4. Electrode modification with G4

A 1 μL of a 102 μM G4 stock solution was diluted with PBS containing 30 μM TCEP to reach the final G4 concentration of 10 μM. In control experiments, a 1 μL of T_{25} was mixed with a 1 μL of 0.5 mM TCEP and 8 μL of PBS to reach the final concentration of 10 μM. For electrode modification, 10 μL of either G4 or T_{25} were spread onto the clean Au electrode surface and left for 2 h immobilization at rt. For immobilization of the covalent hemin-G4 complexes, 5 μL of the hemin-G4 dialyzate were placed on the electrode surface and left for 2 h at rt. After DNA immobilization, all electrodes were kept for 30 min in a 2 mM mercaptohexanol solution in PBS, rinsed in PBS, and dried in a N_2 flow.

2.5. Covalent coupling of hemin to MC_6NH_2-modified gold electrode

The clean gold electrodes were modified by casting 5 μL of 2 mM MC_6NH_2 in PBS and leaving them for 1 h at rt. Then, the electrodes were washed in PBS and immersed in the mixture of hemin, sulfo-NHS and EDC (final concentrations of 1, 100 and 5 mM), respectively, for 3 h at rt. After that, the electrodes were rinsed in PBS and dried in a N_2 flow.

2.6. Electrochemical measurements

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV, modulation amplitude 25 mV) were performed in a standard three-electrode electrochemical cell connected to a μAutolab potentiostat