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Electrocatalytic activity of DNA on electrodes as an indication of hybridisation

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Abstract

Electron transfer between metal electrodes and ferro/ferricyanide is completely suppressed at low ionic concentration. We describe here a new phenomenon related to this reaction: an immobilisation of thiolated single-stranded DNA on gold electrodes retains this activity at low ionic strength up to the level corresponding to the high ionic strength. In contrast, a hybridisation of the complementary DNA with the thiolated single-stranded DNA followed by a binding onto the electrodes, attenuated the electrocatalytic effect. These effects can be used for discrimination between single-stranded DNA and double-stranded DNA and for semi-quantitative measurement of complementary DNA in a sample.

Keywords: DNA; DNA-sensor; Biosensor; Electrocatalysis; Hybridisation; Olygonucleotide; Gene analysis

1. Introduction

The well-investigated ferri/ferrocyanide redox system, while described in many textbooks on electrochemistry, has a rather complicated reaction mechanism and is catalysed by cations [1–4]. In fact, no electrochemical activity is observed at low ionic strength. A mechanism of this effect is still unknown. We describe here a finding that the ferri/ferrocyanide system displays electrochemical activity even at low ionic strength if thiolated single-stranded DNA (ssDNA-SH) is immobilised on the electrode. If, however, the ssDNA-SH is hybridised first and then bound to the electrode, the electrocatalytic activity is diminished significantly. Hence, electrochemical measurements of the DNA modified electrodes in the presence of ferri/ferrocyanide resulted in characteristic signals enabling not only discrimination between single stranded DNA and double-stranded DNA (dsDNA), but also a quantification of the unlabelled complementary DNA in a small volume. This new phenomenon of the intrinsic electrocatalytic activity of ssDNA-SH can be used as the basis for direct and simple label-free electrochemical assay for detection of hybridisation events in single sensors or in future electronic DNA-arrays for multiplex gene analysis.

Numerous assays were suggested for detection of DNA (reviewed in [5,6]); they include for example fluorescence [7], surface plasmon resonance [8,9], acoustic transduction [10,11] and electrochemical methods [12–20]. While fluorescent hybridisation detection is known since many decades, electrochemical approaches were developed mainly during the last few years [21–23]. These techniques are based mainly on DNA-labelling by electrochemically active compounds or by enzymes catalysing a formation of electrochemically detectable substrates, strong binding of some electrochemically-active compounds to dsDNA. We describe here the new phenomenon of intrinsic electrocatalytical activity of DNA in electron transfer between gold electrode and ferri/ferrocyanide and application of this finding in hybridisation assay.

2. Experimental

5′-HS-$(CH_2)_6$-modified 14-mer oligonucleotide of the sequence GCA AAG GGT CGT AC (P1), 5′-HS-(CH2)6-

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modified 26-mer oligonucleotide of the same sequence but with 12 T-spacer TTT TTT TTT TTT GCA AAG GGT CGT AC (P2) and the 14-mer DNA complementary to P1 were synthesised and provided in lyophilised forms by Thermo Hybaid, Interactiva Biotechnologie GmbH, Ulm, Germany. All oligonucleotides were purified by HPLC or FPLC and their molecular weights were confirmed by MALDI-TOF analysis. The oligonucleotides were dissolved in an appropriate volume of pure water resulting in DNA concentrations of 100 μM, which were further divided into 50-μl aliquots and stored frozen under argon.

Thin-film gold electrodes were prepared by UV photolithography. The macroscopic electrode area was about 0.40 mm^2. The purity of the Au sputtering target was 99.98%. The gold layers display polycrystalline structure with preferred orientation of their grains in the [1 1 1] direction; the surface roughness being less than 10 nm. Special cleaning treatment of the electrodes included ultrasonication in $CHCl_3$/ethanol (1/1, v/v) for 5 min, water for 5 min, the electrodes were further cleaned by exposure to hot H_2O_2/H_2SO_4 mixture (1:3, v/v) for 5 min. Then the electrodes were rinsed thoroughly with double-distilled water and dried under nitrogen stream. Quality control of the electrodes was performed voltammetrically using a solution containing 10 mM $K_3Fe(CN)_6$, 100 mM KCl and 1 mM HEPES buffer, pH 7.0.

Thiol-linked DNA duplexes (dsDNA-SH) were prepared by mixing equimolar amounts (unless otherwise specified) of ssDNA-SH and the complementary oligonucleotide, and hybridised in 5 mM phosphate buffer (pH 7.0) with 50 mM KCl for 10 min at 22 °C. Hybridisation was confirmed by agarose gel electrophoresis. The immobilisation was performed by deposition of 1 μl droplets of corresponding solutions of ssDNA-SH or dsDNA-SH in 50 mM KCl, 5 mM phosphate buffer (pH 7.0) onto gold electrodes and subsequent incubation for 3 h in the high humidity chamber to prevent evaporation. Then the electrodes were rinsed thoroughly with 1 mM HEPES buffer (pH 7.0) and used for electrochemical measurements. The binding of dsDNA-SH and ssDNA-SH to gold electrodes was independently tested by using ^{33}P-labeled olygonucleotides.

Electrochemical measurements were performed in 10-ml volume using the AUTOLAB Electrochemical Workplace (EcoChemie) with a conventional three-electrode arrangement. A saturated calomel electrode (SCE) and a platinum wire were used as the reference electrode and auxiliary electrode, respectively. Unless otherwise indicated, the electrochemical measurements were performed in the solution containing 10 mM $K_3Fe(CN)_6$ and 1 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer, pH 7.0, under argon atmosphere after 10 min of deaeration. The sweep rate of the cyclic voltammetry was 100 mV/s and the voltammograms of the second cycle were presented. Impedance measurements were performed in the frequency range from 1 Hz to 100 kHz. Differential pulse voltammetry was performed at a scan rate of 50 mV/s, pulse amplitude of 30 mV and pulse width of 50 ms. Electrode potentials are indicated vs. SCE. All electrochemical measurements, as well as DNA deposition and DNA hybridisation, were performed at room temperature (21–22 °C).

3. Results and discussion

The catalytic effect of ionic strength on the redox behaviour of ferri/ferrocyanide is shown in Fig. 1a and b, curve 1. Voltammetric peaks of oxidation and reduction observed at relatively high ionic strength of the supporting electrolyte (100 mM KCl, 1 mM HEPES, pH 7.0), disappear completely at low ionic strength (the same solution but without KCl). These effects correspond to the electrocatalytical effect of cations which was firstly reported in 1960 [1] and explained by the suggestion that the formation of the activated complex of ferri/ferrocyanide requires its association cations [2–4]. We have observed a new effect related to this phenomenon: an immobilisation of ssDNA-SH on the gold electrodes restored the electrochemical activity of ferri/ferrocyanide at low ionic strength (Fig. 1b, curve 2). The immobilisation was performed through thiolated spacer linked to the 5′-end of the DNA, the thiol group provides a strong binding to gold electrodes [24]. The electrocatalytic effect of ssDNA-SH was similar to the effect of potassium cations at 100 mM: it leads to approximately the same values of redox peaks on the voltammogram as those in the presence of 100 mM KCl (Fig. 1). ssDNA-SH was not present in the electrochemical measuring solution; therefore, the electrocatalytic effect must be due to processes at the interface. A similar experiment with single-stranded DNA without the thiol group showed virtually no increase of the electrochemical activity of ferri/ferrocyanide at low ionic strength. Also, immobilisation of another acidic compound with a thiol group (3-mercaptopropionic acid) but without the DNA chain did not result in any increase in the electrochemical activity at low ionic strength. Therefore, neither weak non-specific DNA adsorption nor immobilisation of the thiol without single-stranded DNA can provide the electrocatalytic effect.

The ssDNA-SH mediated electrocatalytic activity was observed for both oligonucleotides studied—a 14-mer oligonucleotide (P1) and a 26-mer oligonucleotide (P2). Besides ferri/ferrocyanide, three other redox active substances were tested: ferrocenecarboxyaldehyde, ferrocenemonocarboxylate and hexaammineruthenium (III). However, in contrast to the ferri/ferrocyanide, neither the suppression of their electrochemical activity at low ionic strength nor the electrocatalytical effect in the presence of ssDNA-SH was observed. At the pH value used in the experiments, these redox markers have different electric charges, but both ferri/ferrocyanide and ferrocenemonocar-

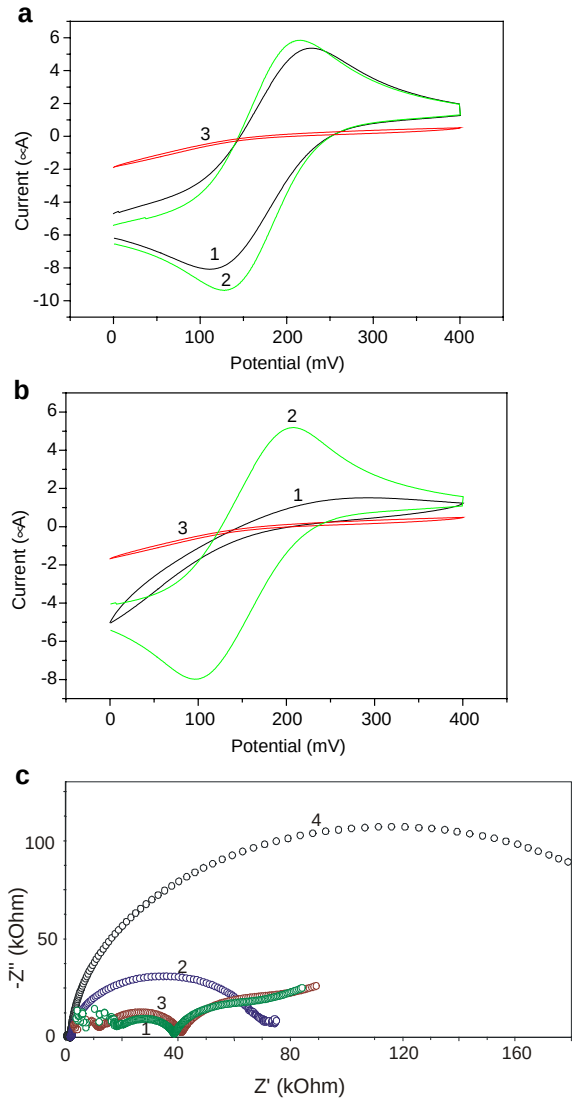

Fig. 1. Cyclic voltammograms (a,b) and impedance spectra ($-Z''$ vs. Z') (c) measured with bare gold electrode, ssDNA-SH modified gold electrode and dsDNA-SH modified gold electrode. Curves in panel (a): bar gold electrodes (1), ssDNA-SH modified gold electrode (2) and dsDNA-SH modified gold electrode (3) being measured in the electrolyte: 100 mM KCl, 10 mM $K_3Fe(CN)_6$, 1 mM HEPES (pH 7.0). Curves in panel (b): bar gold electrodes (1), ssDNA-SH modified gold electrode (2) and dsDNA-SH modified gold electrode (3) in the electrolyte: 10 mM $K_3Fe(CN)_6$, 1 mM HEPES (pH 7.0). Curves in panel (c): bar gold electrodes in 100 mM KCl, 10 mM $K_3Fe(CN)_6$, 1 mM HEPES (pH 7.0) (1) and in 10 mM $K_3Fe(CN)_6$, 1 mM HEPES (pH 7.0) (2); ssDNA-SH (3) and dsDNA-SH modified (4) gold electrodes in 10 mM $K_3Fe(CN)_6$, 1 mM HEPES (pH 7.0).

boxylate are negatively charged. Therefore, the electrocatalytic effect of ssDNA-SH cannot be explained by simple electrostatic interactions and is probably a unique property of ferri/ferrocyanide redox marker. It accords to the interpretation [2] that the electrocatalytic effects of cations cannot be explained in terms of electrical double layer effects.

The electrocatalytic effect of ssDNA-SH was further investigated by impedance spectroscopy, the results are presented in Fig. 1c as Nyquist plots. At high frequencies, where the diffusion component of impedance is negligible and the impedance spectra can be fitted by a semicircle, the diameter of the semicircle can be assumed to be corresponding to the electron transfer resistance. This resistance was about 40 kΩ for bare gold electrode at 100 mM KCl (Fig. 1c, curve 1); a decrease of ionic strength resulted in the increase of the resistance of about twice (Fig. 1c, curve 2). Subsequent immobilisation of ssDNA-SH on the electrode decreased the reaction resistance at low ionic strength to the value which is typical for high ionic strength (Fig. 1c, curve 3). Changes of impedance spectra demonstrating the electrocatalytic effect of cations on ferri/ferrocyanide redox reaction were reported in [4].

The similarity of electrocatalytical activity of cations and immobilised single-stranded DNA allows us to suggest that the two effects have a similar mechanism. One can probably exclude a role of electrical conductivity of DNA in the electrocatalytic effect, since only dsDNA displays the conductivity [25,26]. Therefore, based on the detailed investigation of the cationic electrocatalysis performed by Peter and co-workers [2–4], we suppose that also in the case of ssDNA-SH electrocatalysis either cations adsorbed on the immobilised DNA or cationic groups of DNA bases are involved into the formation of activated complex. DNA hybridisation results in low accessibility of these groups to ferro/ferricyanide. Detailed investigation of the mechanism requires further experiments; here we would like only to describe the phenomenon and to show its analytical potential.

Variations of ssDNA-SH concentration (at a fixed adsorption time) in the immobilisation solution prior to the electrochemical measurements led to different levels of the electrocatalytic effects. This concentration-dependence allows one to use the signal amplitude for quantification of ssDNA-SH concentrations (Fig. 2). It is interesting that an increase of the electrocatalytic activity is accompanied by a decrease of the separation potentials between the reduction and oxidation peaks, thus indicating that the electrochemical reaction becomes more reversible.

In contrast to ssDNA, an adsorption of dsDNA formed by pre-hybridisation of ssDNA-SH with its complementary oligonucleotide did not display electrochemical activity of ferri/ferrocyanide on gold electrodes at low ionic strength (Fig. 1b, curve 3). An investigation of electrochemical impedance showed an increase of the reaction resistance up to more than 200 kΩ (Fig. 1c, curve 4). This reaction resistance was even much higher than that of the bare gold electrodes (curves 1 and 2 in Fig. 1c). Therefore, this blocking effect cannot be explained only by a suppression of the electrocatalytic activity of single-stranded DNA, most probably this is caused by formation of a densely packed insulating layer near the electrode blocking the access of redox active compounds to the electrode surface. An increase of the

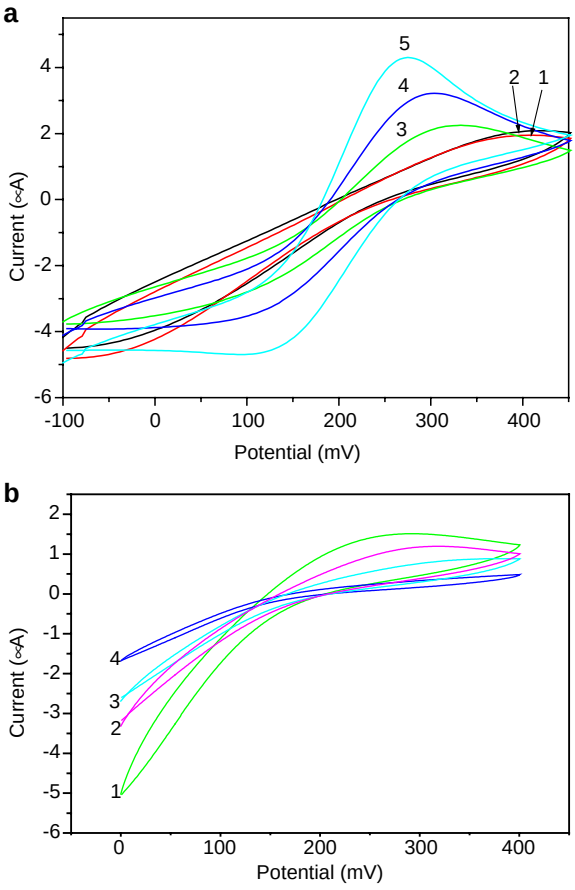

Fig. 2. Cyclic voltammograms of gold electrodes coated by ssDNA-SH (a) or dsDNA-SH (b) as measured in 10 mM $K_3Fe(CN)_6$, 1 mM HEPES (pH 7.0). The concentrations of ssDNA-SH in the coating solution were zero (curve 1), 0.2 μM (curve 2), 2 μM (curve 3), 10 μM (curve 4) and 20 μM (curve 5). The concentrations of dsDNA-SH in this solution were zero (curve 1), 0.2 μM (curve 2), 2 μM (curve 3) and 10 μM (curve 4).

surface density of dsDNA obtained by increasing its concentration in the immobilisation solution during the constant adsorption time led to a gradual increase of the reaction resistance and to a further decrease of current in voltammograms (Fig. 2b). Incubation of the gold electrode with 10 μM of dsDNA without thiol linker did not lead to such changes in voltammograms, therefore weak adsorption of DNA without thiol group does not result in this effect. This fact provides a possibility for discrimination between a sequence of interest hybridising with a thiolated oligonucleotide and other non-target DNAs which could be present in the sample.

The blocking of electron transfer between gold electrode surface and redox mediator by the dsDNA monolayer at high ionic strength has been documented in the literature [12,13]. Recently, it has been used for indication of the melting point of immobilised dsDNA [27]. This phenomenon is, however, less suitable for sequencing purposes because it is impossible to distinguish between signals from bare electrodes and ssDNA-modified electrodes. Such a differentiation is important to distinguish a failure of ssDNA immobilisation on the electrode and the absence of a sequence-matched target. The phenomenon of electrocatalytical activity of ssDNA described here allows us to solve this problem.

The experimental data shown in Fig. 3 demonstrate that the new method provides not only detection but also quantification of specific nucleotide sequences: the concentration of the complementary target in the immobilisation solution varied while the concentration of ssDNA-SH was fixed at 10 μM. The mixing ratios of these nucleotides (complementary target to ssDNA-SH resulting in hybridisation) were 0:1, 0.2:1, 0.5:1 and 1:1. Subsequent electrochemical tests in the presence of $K_3Fe(CN)_6$ demonstrated that the electrochemical activity decreased by increasing the concentration of the target DNA.

Detection limit for 14-mer oligonucleotides was estimated as about 1 μmol/l, it is defined by the binding constant and can be certainly decreased by using of longer oligonucleotides. This will compensate also decrease of the

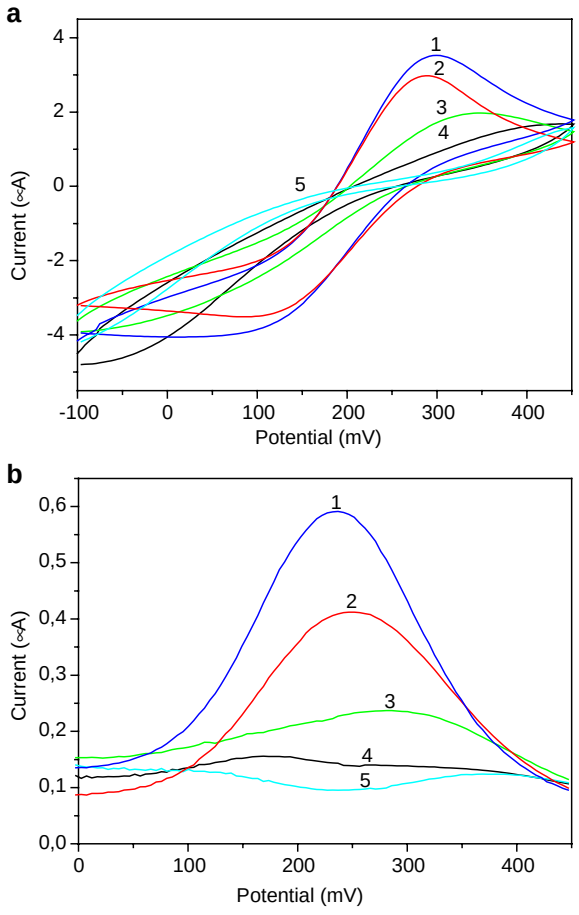

Fig. 3. Dependence of electrochemical signals measured as cyclic voltammetry (a) and differential pulse voltammetry (b) on the concentration of complementary DNA. The gold electrodes were modified by adsorption from the pre-mixed solution consisting of 10 μM ssDNA-SH (P1) and zero (curve 1), 2 μM (curve 2), 5 μM (curve 3) or 10 μM (curve 4) of the complementary DNA. The curve 5 indicates a control experiment with 10 μM of complementary DNA without ssDNA-SH.

binding constant and melting point caused by measurements at low ionic strength [27,28]. The minimal sample volume is limited only by a mechanical possibility to mix two liquids and to bring them onto the electrodes. We have done it with one microlitre droplets (i.e. with 1 pmol of DNA); one can expect that integration of this approach with a microfluidic handing system and with a target-amplification system (i.e. polymerase chain reaction) can provide a chip-based array for ultrasensitive multiplexed DNA assay in a submicrolitre volume.

4. Conclusion

In contrast to most other electrochemical assays for gene analysis, the present approach for DNA detection does not require any toxic electrochemically active intercalators [29–32] or chemical labels [32–35]. Since hybridisation occurs in the bulk phase, typical difficulties caused by the hybridisation on the surface are overcome. We anticipate that further optimisations of hybridisation conditions, of surface functionalizations [36], as well as measurements with a differential configuration or/and in non-equilibrium conditions [37], will improve the specificity as well as sensitivity of this approach.

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