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Label-free electrochemical aptasensor for cytochrome *c* detection using pillar[5]arene bearing neutral red

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ABSTRACT

Novel electrochemical aptasensor toward cytochrome *c* (Cyt *c*) has been developed on the base of glassy carbon electrode (GCE) modified with electropolymerized neutral red (Poly-NR) and decacarboxylated pillar[5]arene (P[5]A-COOH) bearing terminal neutral red (NR) and aminated aptamer specific to Cyt *c*. Addition of Cyt *c* resulted in decrease of the cathodic peak current of NR on cyclic voltammogram due to suppression of the electron exchange between reduced and oxidized NR forms in the surface layer. The implementation of Cyt *c* in the surface layer was confirmed by scanning electron microscopy (SEM), atomic force microscopy (AFM) and electrochemical impedance spectroscopy (EIS). Depending on the content of the surface layer and assembling protocol, the limits of detection (LODs) varied from 0.02 to 1.0 nM and linear range of concentrations was within three orders of magnitude. Interfering influence of some proteins and polyethylene glycol was characterized. The aptasensors developed can find application in detection of Cyt *c* as apoptosis agent in blood serum. This has been partially validated in model blood serum mimicking the ionic composition of the plasma.

1. Introduction

There is an urgent need in the development of simple and reliable biosensors devoted to the detection of biomolecules that play significant role in medical diagnostics and therapeutic treatment [1]. The introduction of modern biosensor technologies expands the number of such biomarkers and is considered as promising alternative to expensive and sometimes time consuming laboratory tests. Further progress in this area is mainly directed to the development of new technologies for the detection of specific interactions between an analytes and biorecognition element to reach faster and more sensitive response [2]. Aptamers as new recognition elements obtained by combinatorial chemistry from random DNA library and selected against targets by affine chromatography have recently found increased attention in medical diagnostics and targeted therapy [3]. Aptamer based biosensors called as aptasensors have been reported for the detection of various species, e.g., thrombin [3,4], mycotoxins [5,6], lysozyme [7], dopamine [8], prions [9], cancer biomarkers [10,11], cells [12] and viruses [13]. The determination of other analytes as well as the prospects of application of aptasensors in various areas were recently summarized in reviews [14–16].

Cyt *c* is an electron-carrying mitochondrial protein that is used in various electrochemical sensors as redox mediator for the detection of nitric oxide [17], nitrite [18] and hydrogen peroxide [19,20]. Besides, Cyt *c* biosensors were successfully applied for the detection of superoxide radical and subsequent assessment of antioxidant capacity [21–23]. The participation of the Cyt *c* molecules in the electron transfer reactions is also considered as a model of similar redox paths in biological membranes [24]. Regarding diagnostic significance, the release of mitochondrial Cyt *c* into the cytoplasm indicates the cell apoptosis [25]. The monitoring of the Cyt *c* release was suggested to use for the screening of anti-cancer drugs [26]. Decrease in the Cyt *c* redox activity was successfully applied for the sensitive detection of anti-respiratory poisons [27]. The

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determination of Cyt *c* in biological samples is commonly performed with immunoassay techniques and electrophoresis [25,28]. A limited number of aptasensors have been developed for Cyt *c* detection on sub-nanomolar level [29–31]. High sensitivity of such aptasensors is achieved by additional amplification steps based on bio-bare code technology [29] or sandwich immunoassay with anti-Cyt *c* antibodies [30]. Being sensitive, the approaches described require sophisticated equipment and time and labor consuming steps of the sample treatment and measurement protocol.

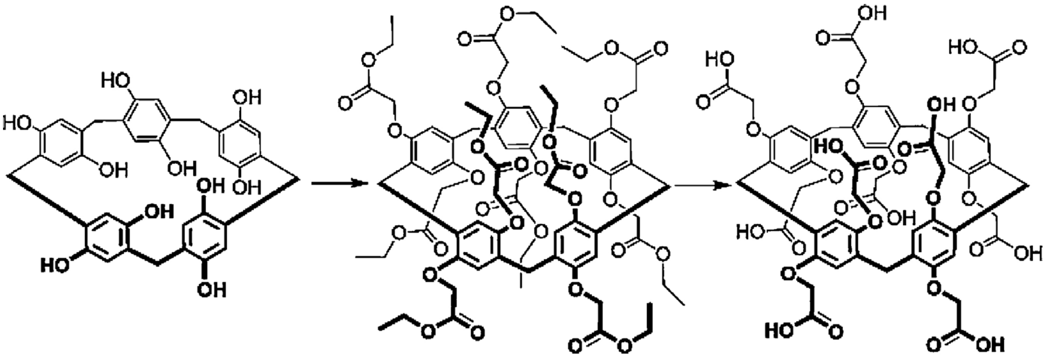
Recently we have proposed the aptasensor assembly based on the use of polycarboxylated thiacalix [4] arene bearing neutral red as redox indicator [32,33]. The introduction of an aptamer in the surface layer followed by analyte binding resulted in limitation of the electron exchange recorded by direct current voltammetry and electrochemical impedance spectroscopy (EIS). New concept of the aptasensor design was proven by detection of thrombin, aflatoxin B1 and ochratoxin A in sub-nanomolar range of their concentrations.

In this work, the above concept was extended by the use of a novel macrocyclic carrier, i.e., polycarboxylated pillar [5] arene which offers greater possibilities for the attaching the reactant and electric wiring of redox centers in the biosensor assembly.

2. Materials and methods

2.1. Reagents

4,8,14,18,23,26,28,31,32,35-Deca(carboxymethoxy)pillar [5] arene (P[5]A-COOH) was synthesized from the unsubstituted pillar [5] arene by consecutive treatment with ethyl bromoacetate in acetonitrile followed by hydrolysis of esteric groups with KOH in aqueous THF as described elsewhere [35] (1).



(1)

After that, the product was filtered and washed several times with deionized water. The structure of P[5]-COOH and its purity were characterized by ^{1}H and ^{13}C NMR, IR-spectroscopy, MALDI-TOF mass-spectrometry and elemental analysis. Product yield: 83%. Mp: 243 °C. ^{1}H NMR (CDCl$_3$) δ_H ppm (*J*/Hz): 3.74 (s, 10H, —CH$_2$—), 4.41 (d ABsystem, 10H, $^2J_{HH}$ = 16.0 Hz, O—CH$_2$C(O)—OH), 4.70 (d ABsystem, 10H, $^2J_{HH}$ = 16.0 Hz, O—CH$_2$C(O)—OH), 7.10 (s, 10H, ArH), 12.94 (br. s., 10H, —C(O)—OH). ^{13}C NMR (CDCl$_3$) δ_C ppm: 170.47; 148.46; 128.07; 114.24; 65.08; 28.58. IR ν cm^{-1}: 3051 (—C(O)—OH), 2911 (—CH$_2$—), 1730 (—C(O)—OH), 1209 (Ph—O—CH$_2$—). MALDI-TOF MS: calculated [M$^+$] *m/z* = 1190.24, found [M+Na]$^+$ *m/z* = 1213.2. Found: C, 55.63; H, 4.18. Calculated for $C_{55}H_{50}O_{30}$: C, 55.47; H, 4.23.

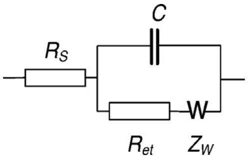
Aminated aptamer 5′-NH$_2$-CCG TGT CTG GGG CCG ACC GGC GCA TTG GGT ACG TTG TTG C-3′ first described for Cyt *c* binding in [29] was synthesized by Thermo Fisher (Germany). Cyt *c* from horse heart, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (NR), 2-(*N*-morpholino)ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), *N*-hydroxysuccinimide (NHS), lysozyme, polyethylene glycol and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Germany).

All the reagents were of analytical grade. Millipore® water was used for the preparation of the working solutions. All the other reagents were of analytical grade and used without additional purification. Millipore® water was used for the preparation of working solutions.

2.2. Electrochemical measurements

Voltammetric measurements were performed in DC mode with the potentiostat/galvanostat AUTOLAB PGSTAT 302N (Metrohm Autolab b.v., the Netherlands) at 23 ± 2 °C in the 15 mL working cell. The three-electrode system consisted of working glassy carbon electrode (GCE, area: 1.67 mm^2), double-junction Ag/AgCl reference electrode (Metrohm Autolab) and Pt wire as a counter electrode. The pH value of working solutions was measured with digital pH-meter-ionometer “Ecotest 001” (Econix-Expert, Moscow, Russia). All the electrochemical measurements were performed in phosphate buffer solution (PBS) containing 187 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$ and 1.76 mM KH$_2$PO$_4$, pH 7.0

The EIS spectra were recorded in the presence of 0.01 M K$_3$[Fe(CN)$_6$] and 0.01 M K$_4$[Fe(CN)$_6$]. The amplitude of the applied sine potential was 5 mV and the frequency varied from 100 kHz to 0.04 Hz with a sampling rate of 12 points per decade. The calculations of the capacitance and the charge transfer resistance were made with the NOVA software (Metrohm Autolab) by the fitting procedure corresponded to the Randles equivalent circuit (2).



(2)

Here R_S and R_{et} are the electrolyte and electron transfer resistances, Z_W is the Warburg impedance and *C* is the capacitance of the electrode surface/solution interface. The dimensionless “roughness” index *n* was higher than 0.88 in all the experiments, so that pure capacitance *C* could be used instead of the constant phase element.

2.3. Scanning electron microscopy (SEM) and atomic force microscopy (AFM)

SEM images were recorded with the field emission scanning electron microscope Merlin^{TM} (Zeiss, 30 kV, 300 pA). The GCE was