Specific Picomolar Detection of a Breast Cancer Biomarker HER-2/neu Protein in Serum: Electrocatalytically Amplified Electroanalysis by the Aptamer/PEG-Modified Electrode

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Specific and sensitive electroanalysis of blood-circulating protein cancer biomarkers is often complicated by interference from serum proteins nonspecifically adsorbing at the biosensing interface and masking specific reactions of interest. Here, we have developed an electrocatalytically amplified assay for specific and sensitive analysis of human epidermal growth factor receptor-2 (HER-2/neu, a protein cancer biomarker over-expressed in breast cancers) that allows us to avoid both the interference from bovine serum albumin (BSA) and electrocatalytic amplification of the signal stemming from the specific aptamer–HER-2/neu binding. A HER-2/neu-specific thiolated aptamer sequence was co-adsorbed on gold together with a C11 alkanethiol bearing two ethylene glycol (EG) head groups that prevented non-specific adsorption of BSA. On such layers, electrochemical reduction of a ferricyanide redox indicator is inhibited and is shown to be electrocatalyzed by methylene blue electrostatically interacting with negatively charged HER-2/neu. The electrocatalytic signal increased upon HER-2/neu binding to the aptamer, which allowed \( \times 10^{-12} \)–\( \times 10^{-8} \) M HER-2/neu detection in 1% serum, being practically applicable for clinical testing. The developed strategy can be considered as general and applicable for the electroanalysis of other blood-circulating proteins once the electrostatic compatibility between the protein and redox probe is established.

1. Introduction

Simple and robust methodological approaches for highly specific and sensitive analysis of physiological fluid-circulating protein cancer biomarkers are crucial for the development of non-invasive point-of-care screening tests for prognosis of cancers and continuous monitoring of individual responses to cancer treatment therapies.[1] Electrochemical aptamer-based assays offer such possibility,[2] including direct analysis in blood serum with redox-labelled aptamer folding beacons.[3] Electro-analysis of the protein-aptamer binding is also possible with more cost-effective electrochemical approaches based on soluble redox indicators[4] that allow avoiding a quite expensive labelling of the aptamer sequence with redox probes.[5] However, such assays often suffer from interference from serum proteins, such as serum albumins, due to their non-specific interfacial adsorption producing false positive signals even in the absence of the analyzed protein.[6] Therewith, the outcome of the assay essentially depends on the design of the biorecognition interface.

In most routinely used approaches, an aptamer sequence is tethered to the electrode through the appropriate linker, and the protein binding to the aptamer is monitored by following the variations in electrochemistry of a soluble redox indicator, which electron transfer (ET) reactions may be either accelerated or impeded by protein binding.[6–7] The most popular redox indicator used hitherto is a ferri-/ferrocyanide redox couple, which electrochemistry is sensitive both to the protein charge and extent of the interfacial blocking.[8] Such assays are simple and robust, and in theory can be used for analysis of any protein once the appropriate aptamer is selected.

Motivated by this research and our previous work on pM analysis of urokinase plasminogen activator (uPA) at the RNA aptamer-electrode, with methylene blue (MB) as a soluble redox indicator,[7] we aimed to use the same approach for electrochemical detection of the Human Epidermal growth factor Receptor-2 (HER-2/neu) protein. HER-2/neu is a 185 kDa transmembrane glycoprotein over-expressed in 25–30% of breast cancers that are characterized by aggressive growing and spreading of the tumors and, as a rule, have poor clinical prognosis.[8] HER-2/neu positive cancers require targeted therapies and thus continuous monitoring of the HER-2/neu levels in response to anticancer treatment. However, the clinical assessment of the HER-2/neu state is currently performed by methods that are not well suited for continuous monitoring and rely mostly on biopsies (either immunohistochemistry or fluorescent-in-situ-hybridization).[9] Electrochemical detection of blood-circulating HER-2/neu, whose levels in healthy individuals range between \( \times 10^{-11} \) and \( \times 10^{-10} \) M, with a cancer cut-off level of \( \times 2 \times 10^{-10} \) M (37 \( \mu \)g L\(^{-1}\)),[10] may be a sensitive and specific in vitro alternative for protein detection complementing the current tissue tests.

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Electrochemical analysis of HER-2/neu exploiting a 42 nucleotide long HER-2/neu-specific DNA sequence\textsuperscript{[11]} tethered to gold and either ferricyanide or MB as redox indicators demonstrated a strong interference from BSA: BSA signals were comparable to those detected with HER-2/neu (unpublished data). Another interesting feature was a dependence of the detected current and impedance signals on the protein concentrations, in the HER-2/neu case inconsistent with 1:1 aptamer-protein binding. Those observations indicated a strong non-specific interfacial adsorption of both proteins.

To avoid detrimental effects of non-specific binding, several approaches can be used, such as prior-to-the-assay surface blocking with BSA,\textsuperscript{[5b]} formation of antifouling ternary self-assembled monolayers (SAM),\textsuperscript{[12]} or the use of antifouling polyethylene-glycol (PEG)-blocking SAMs.\textsuperscript{[13]} Among those, the latter appeared to be most attractive. In addition to their ability to prevent non-specific adsorption of proteins, the PEG-SAMs were shown to inhibit the redox reaction of the ferri-/ferrocyanide redox couple but not electrochemical reactions of other redox molecules, such as ferrocenedimethanol or ruthenium hexamine complex.\textsuperscript{[14]} That feature opens a new perspective in the biosensor research, since such SAMs not only prevent the electrode surface fouling by proteins but also allow the development of electrocatalytically amplified assays, in which signals from redox active indicators capable of binding to the targeted proteins can be amplified in the presence of ferro-/ferricyanide. Here, the redox indicator works as an electrocatalyst for the ferro-/ferricyanide transformation. By this, both the sensitivity and specificity of the assay can be improved.

Previously, the catalytic amplification of the ferricyanide reduction by MB intercalating into DNA duplexes forming densely packed monolayers on gold electrodes has been used for discrimination between the perfectly matched and single-nucleotide mismatched DNA hybrids.\textsuperscript{[15]} Ferricyanide electrochemistry was strongly impeded on such compact, negatively charged DNA SAMs, and intercalated into DNA MB mediated ET between the electrode and ferricyanide and operated as a true electrocatalyst for the ferricyanide reaction. Mechanistically, this assay relied on different rates of ET between the electrode and DNA-intercalated MB, differently mediated by the fully complementary and mismatched DNA duplexes.

In the present work, to reach the required specificity and sensitivity of protein analysis, we suggest exploiting the electrocatalytic reduction of ferricyanide by MB on the mixed aptamer/PEG SAM, the efficiency of this reaction being enhanced by the specific HER-2/neu binding. That is a new concept of electrochemical protein-specific aptamer-based assays. Aptamer electrodes were prepared by co-immobilization of the aptamer with a PEG SAM, inhibiting non-specific adsorption of both BSA and HER-2/neu. Both the aptamer and PEG were immobilized on the gold electrode surface via the routinely used alkanethiol linkers, C\textsubscript{6} and C\textsubscript{11}, correspondingly (Figure 1). In contrast to the DNA-mediated ET underlying electroanalysis of DNA mismatches,\textsuperscript{[15]} in our approach we expect that MB molecules differently interacting with the aptamer- and aptamer-HER-2/neu complex will produce direct (unmediated) electrochemical signals. Different electrochemical responses will be in a different manner electrocatalytically amplified in the reaction with ferricyanide and by this allow sensitive and specific analysis of the HER-2/neu protein with a minimized interference from BSA.

2. Results and Discussion

2.1. Ferri-/Ferrocyanide Electrochemistry at Aptamer/PEG-Modified Electrodes

First, the electrochemical behavior of ferricyanide and MB redox indicators on the mixed aptamer/PEG SAM was interrogated (for simplicity, SH-C\textsubscript{11}-(ethylene glycol)\textsubscript{2}-OH used here is further referred to as PEG). Co-adsorption of the DNA aptamer bearing a C\textsubscript{12}-alkanethiol linker and PEG with a C\textsubscript{11}-alkanethiol linker was optimized 1) to avoid competitive desorption of DNA (possessing a shorter alkane chain linker) and 2) ensure both a sufficient for biosensing surface concentration of the aptamer and surface packing of PEG that would inhibit the redox reaction of ferricyanide. As can be seen in Figure 2A, co-adsorption of DNA and PEG resulted in the inhibition of the ferri-/ferrocyanide reaction on the modified electrodes indeed. The effect was most pronounced on comparison with the aptamer-mercaptohexanol system, Figure 2A inset. The potential window of the electrochemical stability of the mixed SAM is however quite narrow, and at sufficiently positive potentials (above 0.5 V) oxidative desorption of thiols occurred, resulting in the increased currents from the ferri-/ferrocyanide couple, then discharging also at the formed islands on the electrode surface.

![Figure 1.](image-url)
free from the adsorbed species (potential scan 1 versus 2, Figure 2A). At potentials lower than $-0.5\, \text{V}$ the reductive desorption of the thiolated DNA started to interfere (data not shown). In further experiments, we restricted the potential window to $0.1\, \text{V}–0.5\, \text{V}$ relevant for studies of the electrocatalytic reduction of ferricyanide.

In the Figure 2A system, a quite high aptamer surface coverage was obtained, reaching in some cases 70–80% of the theoretical monolayer, which might produce steric hindrance for the ligand binding as has been shown in our previous works.\[16\] It would also lessen the antifouling properties of the surface due to its insufficient coverage with PEG. The protocol was further optimized and, as can be seen from Figure 2B, a shorter time of the aptamer immobilization and a longer time for the PEG immobilization allowed both better blocking of the electrode surface against ferricyanide reduction and a decreasing surface coverage of the aptamer. In the Figure 2B system, the aptamer surface coverage reached $4.6 \pm 0.48\, \text{pmol cm}^{-2}$ roughly representing less than 50% of the theoretical monolayer. Such surface concentrations were shown to provide more conformational freedom for protein binding.\[16a\]

Interestingly, under those conditions co-adsorption of the negatively charged DNA and PEG enhanced the inhibition of the ferricyanide reduction compared to pure PEG SAMs (Figure 2B). Though DNA adsorption is well known to impede the ET reaction of the ferri-/ferrocyanide couple, due to the electrostatic repulsion of the negatively charged $[\text{Fe(CN)}_6]^{3--/4-}$ complex by a negatively charged DNA backbone,\[4\] based on the Figure 2A inset data such a synergetic action of co-adsorbents was not anticipated.

### 2.2. Electrochemistry of Methylene Blue at Aptamer/PEG-Modified Electrodes

Next, electrochemical behavior of MB on the aptamer/PEG-modified gold electrodes was studied with $0.1\, \mu\text{M}\, \text{MB}$. This sufficiently low concentration of MB was chosen to minimize the contribution from otherwise solution electrochemistry of MB.\[17\] Experiments were also performed within the $-0.5–0.1\, \text{V}$ potential window, where the SAMs were electrochemically stable.

At scan rates lower than $1\, \text{V s}^{-1}$, a well-developed couple of redox peaks with a mid-potential at around $–0.22\, \text{V}$ could be followed (Figure 3). At higher scan rates, due to the increasing peak separation the reduction of MB appeared to be not completely accomplished, and the corresponding oxidation peaks could be correlated only with a part of MB molecules that underwent reduction. Consistently, the MB peak currents showed a linear dependence on the potential scan rate, characteristic of ET in the adsorbed state,\[18\] only at scan rates below $2\, \text{V s}^{-1}$ (Figure 3, inset).

Therewith, the full-width-at-half maximum of the MB peaks approached $100\, \text{mV}$, being closer to the values expected for a one electron transfer reaction.\[19\] Generally, MB undergoes a $2\, \text{e}^-/\text{H}^+\, \text{reaction, but in a number of systems, such as MB intercalated into DNA}\[17\] or attached to the dendrimer structures,\[20\] a $1\, \text{e}^-\, \text{transfer reaction has been demonstrated. It appears to be the case in our system as well and might imply a specific MB-aptamer of MB-PEG interactions stabilizing the intermediate reduction state of MB.}$

Despite a quite low ET rate constant of $0.62\, \text{s}^{-1}$, determined according to Laviron’s formalism from the CV peak potential separation at low scan rates,\[18\] MB efficiently electrocatalyzed the reduction of ferricyanide starting from the potentials of the MB redox transformations, both at low and high scan rates (Figure 4). It was a question if the MB interactions with such proteins as HER-2/neu and BSA could result in sufficient variations of the observed electrocatalytic currents. Based on their $\text{pI}$, both proteins are negatively charged at $\text{pH} 7$ used for studies (see Experimental section), and their binding to the electrode surface might have a dual effect: 1) the additional blocking of the surface by negatively charged bulky molecules should restrict the surface accessibility for the negatively charged ferricyanide and thus inhibit the catalysis; 2) additional adsorption of the positively charged MB on both HER-2/neu...
Figure 5. Representative CVs recorded with the aptamer/PEG-modified gold electrode in 20 mM PBS, pH 7, containing 1) 0.1 μM MB and 2) 0.1 μM MB and 0.2 mM K₃Fe(CN)₆, scan rate 0.1 V s⁻¹ (main figure) and 5 V s⁻¹ (inset).

Figure 4. Representative CVs recorded with the aptamer/PEG-modified gold electrode in 20 mM PBS, pH 7, containing 1) 0.1 μM MB and 2) 0.1 μM MB and 0.2 mM K₃Fe(CN)₆, scan rate 0.1 V s⁻¹ (main figure) and 5 V s⁻¹ (inset).

To understand if we can discriminate between specific binding of HER-2/neu and non-specific interfacial adsorption of BSA, the aptamer/PEG-modified electrodes were incubated in solutions with different concentrations of BSA and HER-2/neu, and CVs were recorded with the protein-reacted electrodes in solutions containing both MB and ferricyanide (Figure 5). The CV responses of the aptamer/PEG-modified electrodes to BSA and HER-2/neu were analyzed at different scan rates, and the most robust signals were observed at high scan rates. We ascribe that to the minimal time of exposure of the electrode to negative potentials. That should minimize the electrostatic repulsion between DNA/DNA-protein complexes and the electrode surface induced by the electric field. In the absence of such repulsion a direct electrical communication between the electrode and the protein-adsorbed MB working as an electrocatalyst for the ferricyanide reduction reaction is facilitated and allows unambiguous detection of the protein binding.

As can be seen in Figure 5A, at all interrogated potentials the aptamer/PEG modified electrodes demonstrated a sufficient tolerance to nonspecific adsorption of BSA. Though the currents first increased in response to 1 pM BSA, with a further increasing BSA concentration they drop down, approaching the original level (Figure 5A, inset). Along with that, they were essentially lower than the electrocatalytic signals stemming from the HER-2/neu binding to the aptamer-modified electrode surface, readily responding to 1 pM HER-2/neu and demonstrating particularly high currents within the clinically important 10⁻¹⁰ and 10⁻⁹ M range; those currents could be calibrated versus the concentration of the protein (Figure 5B). Thus, we can assume that though the aptamer/PEG SAM did not allow us to totally avoid non-specific adsorption of BSA (which is an expected result, since BSA adsorption depends on the PEG packing density and was shown to be suppressed only on quite compact SAMs, for which the PEG coverage exceeded 60 %), BSA adsorption was significantly inhibited, and that allowed discrimination between nonspecific BSA and specific HER-2/neu binding to the aptamer/PEG-modified surface.

Analysis of the electrocatalytic responses from both proteins normalized for the background signals (Figure 6) shows that due to the electrocatalytic amplification of the MB signals associated with the specific biorecognition of the HER-2/neu protein by the aptamer, the signals from non-specific interfacial adsorption of BSA are minimized to the levels of 3–22 %, depending on the protein concentration range. Those results demonstrate a significant improvement in the specificity of the protein binding compared to the aptamer-electrode system with mercaptohexanol as a blocking agent, on which signals from HER-2/neu and BSA were undistinguishable. Thus, the combination of the aptamer and PEG SAMs can be productively used indeed for sensitive and specific electroanalysis of proteins.
Storage stability of the aptamer-PEG electrodes was investigated with similarly prepared aptamer-PEG electrodes, dried in the N₂ flow and stored at 4 °C in a dry state.[23] In general, the detected analytically valuable signals dropped ca. 50% compared to those detected with freshly prepared electrodes, which may be connected with a partial desorption of the thiolated aptamer (Figure 7A). However, the apparent sensitivity of analysis did not change so dramatically, not the least at the expense of the calibration curves becoming steeper (Figure 8).

![Figure 7](image1.png)

Figure 7. Representative CVs recorded with the aptamer/PEG-modified gold electrodes (black line) before and (colored lines) after additions of 1, 10, 100 pM, 1 and 10 nM of HER-2/neu, A) after 5 days of the electrode dry state storage and B) with freshly prepared electrodes in 1% serum. Potential scan rate 5 V s⁻¹. Inset: dependence of the current signal change (corrected for the current in the absence of the protein) on the logarithmic concentration of HER-2/neu, derived from the CV currents at (from 1 to 5): -0.1, -0.2, -0.3, -0.4, and -0.5 V.

Finally, a practical electroanalysis of HER-2/neu in serum was performed (Figure 7B). Albumin content in human serum ranges between 0.53 and 0.75 mM,[24] and, based on supplier’s information, is quite similar to that in bovine calf serum used in the current work. Those concentrations are high compared to the concentration levels of HER-2/neu (10⁶ fold difference in concentrations). Both in whole serum and in 10% serum the electrocatalytic signals associated with the HER-2/neu binding were significantly inhibited. In addition, de-aeration of serum-containing solutions resulted in the intense foam formation impeding the analysis as well. Considering that the developed methodology allows 1 pM analysis of HER-2/neu, and the cancer cut-off levels are 100 times higher than this concentration, serum was ultimately diluted 100 times, which allowed detection of HER-2/neu within the 1 pM–1 nM concentration range, the same range as in the buffer solutions.

Along with that, the reproducibility of analysis became worse and the sensitivity of analysis decreased ca. 2.7 fold, if one will relate the slopes of the dependences of the apparent sensitivity of the HER-2/neu detection at different potentials, derived from the CV data (Figure 8). It is worth to stress that those are apparent sensitivities estimated as a difference in the electrocatalytic currents stemming from 10 nM and 1 pM HER-2/neu binding related to the logarithmic concentration difference and the electrode surface area (but not from the slopes of ΔI-log[HER-2/neu] dependences, which are not strictly linear at every potential studied, Figures 5b and 7, insets).

Thus, the suggested simple and label-free methodology allows fast (30 min analysis) and specific detection of HER-2/neu in such physiological medium as serum, i.e. in the presence of albumin and other proteins present in a large excess over HER-2/neu. Compared to other electrochemical approaches developed for sensitive and specific analysis of HER-2/neu such as electrochemical immunoassays and those with aptamers (Table 1), the suggested protocol offers several advantages such as simple and relatively cheap construction of the aptasensor and fast analysis, with the clinically requested sensitivity and selectivity of the detection. Along with that, finer tuning of the system should be done, to make it more robust for analysis in serum, and experimental conditions should be further adjusted to swap fast scan CV for more analytically appropriate method of detection and avoid interference from solution oxygen.

It is important to stress, that further development of protein assays based on the developed concept requires a careful design of the specific protein - redox indicator couples. The studied HER-2/neu-aptamer system demonstrated a remarkable performance, with an electrocatalytically-amplified practically useful increase of the analytical signal upon protein binding, which helps to avoid artefacts connected with possible electrode degradation, such as desorption of thiols from the electrode surface. Here, both studied proteins, HER-2/neu and BSA, bore a negative net charge, which promoted their interactions with positively charged MB – and ensured the increasing electrocatalytic response. If the target protein is positively charged, such as in the case of uPA, the outcome of the assay may be different.[7] For example, uPA protein binding to the aptamer/mercaptohexanol-modified electrodes resulted in the decreasing signals from MB as a redox indicator, the same effect was observed with BSA, both detected at low potential scan rates. That emphasizes the important role the electrostatic compatibility between redox indicator molecules and proteins plays and often underestimated contribution of the electric field differently affecting the adsorption state of differently charged proteins.[7] Therewith, non-specific adsorption of BSA did not interfere (as much as in the current work) with the
specific-binding of uPA to the aptamer electrode and electrochemical signal read-out.

3. Conclusions

The electrocatalytically amplified assay for specific and sensitive analysis of a cancer biomarker protein HER-2/neu developed here allows 1 pM–10 nM HER-2/neu detection, also in serum, which is relevant for clinical applications. The assay represents a new concept for the electrocatalytically amplified detection of proteins at the aptamer-modified electrodes. In the assay, a thiolated HER-2/neu-specific DNA aptamer sequence was co-immobilized on the gold electrode surface together with the thiolated PEG, and that allowed to minimize signals from non-modified electrode, the electrocatalysis enhancement occurred as a result of the increasing number of MB molecules attracted to the negatively charged HER-2/neu-aptamer complex. The demonstrated here new concept for the HER-2/neu protein detection is applicable for electroanalysis of other proteins by the aptamer-modified electrodes, though, it needs further development and practical adjustment for real sample analysis, with a replacement of fast scan voltammetry for more appropriate for clinical analysis differential or amperometric techniques. Therewith, a particular attention should be paid to the electrostatic interactions between the redox indicators, proteins and electrodes, critically affecting the outcome of such assays.

Experimental Section

Materials

A 47-mer 5′-thiol-modified DNA aptamer sequence specific for HER-2/neu: 5′-OH-C6-(S)-S-C6-GCA GCC GTG TGG CAG CCG TGT GGG GCC AGC GGT GTG GGG TT TTT-3′[11] was synthesized by Metabion Int. AG (Martinsried, Germany); it contained an additional dT2 region at the 3′-prime not affecting the aptamer activity but introduced for enhancement of the negative charge on the aptamer sequence. The extracellular domain of human HER-2/ErbB2 protein, a recombinant form expressed in human cells,
lyophilized (referred in this work as HER-2/neu, 626 amino acids sequence, MW 70 kDa, pl 5.8) was purchased from Sino Biological Inc. (Beijing, PR. China). Bovine serum albumin (BSA, MW 66.5 kDa, pl 4.7). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 6-mercapto-1-hexanol (MC6OH), methylene blue (MB), hexaammineruthenium(III) chloride ([Ru(NH3)6]Cl3, RuHex) and all components of buffer solutions were purchased from Sigma-Aldrich (Germany). Fetal bovine calf serum was from Invitrogen, Life Technologies (currently, ThermoFisher Scientifics), Carlsbad, USA, and stored frozen at –20 °C. Potassium ferricyanide (K3Fe(CN)6) was from Merck (Germany) and SH-C11-(ethylene glycol)2-OH (SH-C11-(EG)2-OH) was from ProChimia Surfaces (Poland). All solutions were prepared with Milli-Q water (18 MΩ cm, Millipore, Bedford, MA, USA).

Gold Electrode Pretreatment

Prior to use, gold disk electrodes (2 mm in diameter, CH Instruments, Austin, TX, USA) were pre-treated by consecutive CV cycling (between −0.5 and −1.4 V, 10 cycles, scan rate 50 mVs−1) in 0.5 M NaOH, mechanical polishing on micro-cloth pads (Buehler, Germany) in 1 μm diamond and 0.1 μm alumina slurries (Struers, Copenhagen, Denmark), followed by ultra-sonication in a 1:1 (v/v) ethanol-water mixture for 15 min, and by final electrochemical cleaning in 1 M H2SO4 (10 CV cycles within the −0.3 to 1.7 V range, scan rate 0.3 Vs−1) and 0.5 M H2SO4/10 mM KCl (10 cycles, 0–1.7 V, 0.3 Vs−1). The electrochemically active surface area was estimated from the gold surface oxides reduction peak in 0.1 M H2SO4 related to 390 μC cm−2 as 0.073 ± 0.003 cm2. Prior to use, the clean electrodes were kept in absolute ethanol.

Electrode Modification with DNA

Prior to immobilization, the DNA aptamer sequence was allowed to react with 0.5 mM TCEP for 1 h to reduce disulfide bonds of the linker. For the electrode modification, a 10 μL drop of 5 μM of the DNA aptamer in 5 mM NaH2PO4/Na2HPO4 solution (PBS, pH 7) containing 0.05 M NaCl/ 0.1 M MgCl2 (immobilization buffer was placed on the electrode surface and left for 1 h at RT, covered with a lid. The electrodes were then rinsed with the PBS buffer (for 5 min) and incubated in 0.3 mL of 2 mM SH-C11-(EG)2-OH solution (or, alternatively, 40 mM MC6OH) for 2 h. The modified electrodes were finally rinsed with PBS buffer (5 min) and used in electrochemical measurements.

Analysis of the DNA Surface Coverage

The aptamer surface coverage was estimated according to the method established by Steel et al. using by evaluating the saturating amount of RuHex electrostatically attracted to the aptamer. The response was measured chronocoulometrically (step potential from +0.1 to −0.4 V, pulse period 0.5 s) in 10 mM PBS, pH 7, in the absence and presence of 0.2 mM Ru(NH3)6$^{3+}$. The aptamer surface coverage $I_{HER-2/neu}$ (moles cm$^{-2}$) was then estimated according to Equation (1):

$$I_{HER-2/neu} = (nA \times C_{0.5} \times F \times C_{Cl_{m}})$$

where $m$ is the number of bases in the aptamer sequence, and $z$ is the charge of Ru(NH3)6$^{3+}$.

The average surface coverage of the DNA aptamer was 4.6 ± 0.48 pmol cm$^{-2}$ for the 1 h immobilization time used.

Electrochemical Measurements and Analysis

All electrochemical measurements were performed with a μAutolab electrochemical system (Type III, Metrohm, Utrecht, the Netherlands) equipped with GPES (version 4.9.007) and NOVA (version 1.8.17) software, in a 3 mL three-electrode cell (a 2 mL buffer solution volume). The modified gold electrodes served as working electrodes, and an Ag/AgCl (3 M KCl) electrode and a Pt wire were employed as the reference and counter electrodes, correspondingly. All experiments were performed at RT (22 °C). Cyclic voltammograms (CVs) were recorded in 20 mM PBS, pH 7, with 0.1 μM MB and 0.2 mM K3Fe(CN)6 as soluble redox indicators, if not specified otherwise. Protein binding was carried out by incubating the modified electrodes for 30 min at RT in 20 mM PBS solutions containing different protein concentrations. After incubation, the electrodes were immediately used for electrochemical measurements in the same cell. The ET rates constant, $k_e$, was estimated according to Equation (2), by analysis of CV peak separations not exceeding 200/n mV$^{[19]}$

$$m = \frac{(RT/F)(k_e/nv)}{1}$$

where $m$ is a tabulated parameter that depends on the peak separation, $n$ is the number of electrons involved in ET, and $v$ is the potential scan rate$^{[19]}$. $R$, $T$ and $F$ have their usual meaning. Values of $n$ were determined from the full-width-at-half maximum of the CV peaks equal 62.5/$(1-n)\mu$ mV for the cathodic peak and 62.5/$(1-n)\mu$ mV for the anodic peak$^{[19]}$.

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Conflict of Interest

The authors declare no conflict of interest.
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[22] Comment: the phenomenon may be connected with a zero balance between effects of the ferricyanide repulsion by BSA and change of the MB molecules in electronic communication with the electrode.
[23] Comment: Storage in the buffer solution results in worse results, due to the faster “corrosion” of gold in the presence of O₂ and H₂O, resulting in a faster desorption of thiols.

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