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Direct Reagentless Detection of Affinity Binding of Recombinant His-Tagged Firefly Luciferase with Nickel-Modified Gold Electrode

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Abstract

The direct reagentless electrochemical detection of recombinant firefly luciferase binding with gold electrode modified with nickel ions has been carried out. Self-assembly of molecules of functional disulfide containing chelating moieties on the gold electrode was monitored by contact angle measurements and confirmed by electrochemical data as three times decrease of electric double layer capacitance. Succeeding nickel ions chelating followed by selective binding of target protein were registered by reagentless electrochemical measurements as consistent decreases of double layer capacitance. The quantification of bound protein has been achieved by means of luminescent assay of residual enzymatic activity in solution for electrode recovery. The electrode surface coverage by protein globules has been confirmed by AFM.

Keywords: Direct reagentless detection, firefly luciferase, His-tagged protein, organic disulfide, SAM.

1. Introduction

Immobilization onto plane surface is a first step in the most of studies of proteins functions and structures. This is the case for instance in the fabrication of protein arrays for functional proteomics, including high-throughput analysis of protein-protein and protein-nucleic acid interactions as well as protein-small molecule interactions for drug screening [1-3]. Physical adsorption is the simplest way to link a protein molecule to a solid surface, but this approach is not commonly used for analytical purposes because it produces a deficient surface modification and the high impact of denaturation. In order to avoid these restrictions and to attach the protein molecule to the metal substrate self-assembled monolayers can be used, basically in two ways: (i) chemical modification of the protein with a linking structure that is able to specifically react with the substrate to form a SAM [4, 5]; (ii) perform reactions on the SAM to then catch the biomolecule. The employment of first route is limited by the loss of protein functionality caused by chemical modification as well as the presence of many sites of modification, which leads to disoriented immobilization. Site-selective methods allow the defined attachment of proteins with uniform orientation where the bioactive site is freely accessible for further analysis or application [6, 7]. This approach retains the biological activity of the proteins and maximizes the signal-to-noise ratio and resolution of the array or biosensor. The use of bifunctional agents to bind biomaterial to solid supports is actually greatly employed due to easy of formation of monolayers with reasonable stability even with limited diffusion of the substrate towards the immobilized biological sensing element [8].

The production of recombinant proteins with previously designed aminoacid sequences became a biotechnology routine. Facilitating of the recovery as well as attachment of different featured properties to recombinant protein can be attained by using of peptide sequences genetically grafted onto recombinant protein, i.e. affinity tags [9]. The hexahistidine tag (His₆), is the most commonly used affinity tag for protein recovery because of its small size with minor effect of protein native function and specific interaction with transition metals ions employing immobilised metal affinity chromatography [10, 11]. The imidazole moieties of the tag-modified protein can chelate to the free coordination sites of divalent transition-metal ions, which are themselves immobilized onto solid supports via appropriate chelating agents such as the tridentate iminodiacetic acid (IDA) or the tetradentate nitrilotriacetic acid (NTA) [11]. This protein immobilization is fully reversible on addition of competitive compounds (like imidazole or histidine), or on removal of the metal ion by EDTA complexation.

Self-assembled monolayers (SAMs) of metal-chelating lipids [12-18] or thiol-functionalized chelating agents [10, 11, 19-25] on gold supports have been widely investigated

for the oriented tethering of His-tagged proteins because of the compatibility of gold supports with surface plasmon resonance, which was used for studies of kinetics and thermodynamics of specific binding between the immobilized protein and a target molecules [7, 10, 20, 23, 26-30]. On the other hand gold can be used as an electrode for the electrochemical studies of the immobilized proteins [31], where the mediated electrical communication of an enzyme [25, 32-34] or antibody orientation [35] or an allosteric protein [19, 28] with the gold electrode can be finely tuned through protein orientations on the SAM.

In this work, the specially synthesized organic disulfide with functional dicarboxypyridine moieties (Fig. 1A) was utilized for the nickel-assisted capture of recombinant His-tagged firefly luciferase onto polycrystalline gold electrode. The binding of luciferase with nickel-modified SAM has been registered directly by impedance spectroscopy and confirmed by AFM and optical measurements of residual enzyme activity in solution for electrode surface recovery.

2. Experimental

Materials

All experiments were carried out with Millipore (MilliQ Plus) water. All inorganic compounds were of analytical grade.

Recombinant firefly luciferase N-His₆-luc (Mr 63 kDa) with specific activity of $1,05 \times 10^{13}$ relative light units/mg was provided by Dr. M. Koksharov and Prof. N. Ugarova (M.V. Lomonosov Moscow State University).

Procedures

The synthesis of 1,16-di[4-(2,6-dihydroxycarboxypyridine)]-1,16-dioxa-8,9-dithiahexadecane termed disulfide herein after is described in Supporting Note 1.

Contact angle measurements ($\pm 2^\circ$) have been preceded with horizontal microscope-goniometr MG (Russia).

Electrochemical methods

Voltammetric measurements were carried out using an electrochemical interface microAutolab III (Eco Chemie, Utrecht, Netherlands) and a three-electrode cell with separated compartments. Glassy carbon rod was used as counter electrode. Working gold disk electrodes (diameter of 1 mm) were made from gold wire of 99.99% purity (Goodfellow, Huntingdon, UK) pressed into Teflon tube. The electrodes were mechanically polished with alumina powder (Al₂O₃, 0.05 μ m) up to a mirror finish and then ultrasonicated in distilled water. Prior to use, the

electrodes were treated with piranha solution (3:1 of sulfuric acid to 30% H_2O_2) during 10 minutes. Finally the electrodes were cycled in 0.2 M sulphuric acid in a potential range 0 to 1.45 V at a sweep rate of 20 mV/s until a stable voltammogram obtained. The roughness factor of the electrode, 3.9 ± 0.2 , was determined as the ratio of the true to the geometric surface area, there the true area was found from the charge needed to reduce the chemisorbed oxygen layer in pH 7.0, 0.1 M phosphate buffer using a scan rate of 50 mV/s. An Ag|AgCl electrode in 1M KCl was used as a reference in all experiments.

After pre-treatment the electrodes were rinsed with water and 96% ethanol. Then the electrodes were placed in solution of disulfide for ca. 12 hours. After that the electrodes were rinsed with ethanol and buffer and then placed into electrochemical cell or were treated with modifications.

Impedance measurements were done with Solartron 1255 Frequency Response Analyzer (Farnborough, UK) with a homemade low noise potentiostat in two-compartment electrochemical cell. The frequency range was from 1 Hz to 20 kHz. The working and counter electrodes were placed in the same compartment. The counter electrode in impedance measurements was a platinum tube encircling the working disk electrode.

Gold sheet pretreatment and modification

Silica sheets ($5 \times 5 \times 0.1$ mm) with sputtered gold (thickness of 50 ± 10 nm) were purchased at NT-MDT (Russia). Sheets were annealed by gas-air flame for 5 seconds and cooled under argon stream. Then the sheets were placed into 10 mM disulfide solution in ethanol for overnight at room temperature. Then a half of the modified gold sheet was immersed in a 10 mM Ni_2SO_4 solution in ultrapure water for 1 hour. After washing with ultrapure water modified sheet was placed in 1 μM N-His₆-luc solution in buffer (0.1M KCl, 0.01 M phosphate buffer, pH 7) for 2 hours. Finally, the sheet was carefully washed with large volume of the buffer and ultrapure water. The prepared sheets were carefully blow with argon and kept for several hours in Petri dish at room temperature before AFM measurements with atomic force microscope MultiMode V (Veeco, USA). The modified and unmodified parts of the plate were scanned at least in three different points using tapping mode with cantilever frequency of 314.7 kHz. Silicon cantilever with constant of resilience 0.03 N/m (Ultrasharp CSG, NT-MDT, Moscow) was used as a sonde. The recorded images ($1 \times 1 \mu\text{m}^2$) were corrected by fitting and subtracting of 3rd order polynomial, and then roughness parameters were calculated for two different section of each picture with areas of ca. 270 and 135 nm^2 using NanoScope 7.2 software.

Luciferase activity measurements

N-His₆-luc activity was measured with luminometer FB12 (Zylix Corp., USA) by the maximum of light intensity emitted due to the enzymatic reaction in saturated substrates concentrations. Aliquot of 5 μ L of enzyme solution has been added to the cuvette with 350 μ L 1,7 mM solution of ATP in 50 mM TRIS-acetate buffer solution (2 mM EDTA, 10 mM MgSO₄, pH 7.8).

3. Results and Discussion

Functional SAM on gold surface

The dimeric design of synthesized disulfide with functional moieties for binding of divalent transition-metal ions (Fig. 1A) has been chosen in order to achieve higher complex efficiency by two adjoining functional groups as well as formation of two adsorbed molecules by single adsorption step. The modification of gold sheets surface by disulfide self-assembly has been monitored by contact angle measurements with water droplets (Fig. 1B). The decrease of contact angle with self-assembly in comparison with blank gold showed the hydrophobisation of surface due to the adsorption of disulfide molecules. The short treatment for 1 minute led to significant contact angle decrease due to the fast adsorption of disulfide. Successive assembly led to the formation of the dense and uniform monolayer. The formation of dense monolayer has been achieved after 12 hours of self-assembly, which was utilized for the modification of gold electrodes.

Cyclic voltammetry of ferri/ferrocyanide redox couple at the modified electrodes (data not shown) showed the decrease of redox currents and the increase of peak-to-peak separation comparing with naked gold electrodes that indicated partial blocking of the electrodes surface. The blocking properties of the layer remained during continuous cycling.

The linear sweep voltammogram recorded with disulfide-modified gold electrode in strong basic aqueous solution (Inset of Fig. 1B) presents a typical peak current of reductive desorption of disulfide molecules at -0.95 V (Ag/AgCl) [36-40]. Charge required for desorption and calculated from the reduction peak area with one electron process assumption [41] was used for the estimation of surface concentration of adsorbed disulfide after 24 hours of incubation, which was 1.7 nmols·cm⁻². Taking into account the roughness coefficient of the gold electrodes about 3.9 [41, 42], the value of 0.4 nmols·cm⁻² of disulfide surface concentration was calculated, which is close to theoretical value of 0.8 nmols·cm⁻² for condensed monolayer [36]. Lower than theoretical density can be explained by coulomb repulsion between negatively charged carboxy-groups of the disulfide and larger molecule dimensions. LSV recorded with electrodes modified with non-dense SAM (1.5 hours of assembly) showed smaller peak of reductive desorption corresponding to smaller interfacial concentration of 0.1 nmol·cm⁻².

The electrochemical impedance spectroscopy was utilized for the reagentless monitoring of interfacial properties of gold electrodes. Fig. 2 represents the impedance spectra in complex coordinates of the frequency-normalized admittance. The diameter of semicircle, observed on all spectra, corresponds to the double layer capacitance at the electrode|solution interface. It is seen, that disulfide self-assembly (Fig. 2A) led to 5 times decrease of double layer capacitance due to the extension of the effective thickness of capacitor of the electric double layer and decrease dielectric permittivity of the interface layer [43-45]. Subsequent electrode modifications with nickel ions and protein led to less prominent decreases of double layer capacitance (Fig. 2B) due to the removal of binding from the actual double layer region, which dominates the double layer capacitance.

In order to make a quantitative assessment of changes of electrode interfacial properties due to successive modifications the equivalent circuit analysis has been carried out (see Supporting Note 2). The corresponding simplified Randles circuit (Fig. 2SE) consists of the solution resistance (R_s), which did not vary with different stages of electrode modification, in series with double layer capacitance. Taking into account the roughness and of the electrode surface, the constant phase element (CPE) was utilized instead of pure capacitance. CPE is represented by two fitting parameters: A and ϕ , where the last one varies from 0 to 1 (when ϕ is equal to 0, the CPE behaves as a pure resistance, when $\phi = 1$, the CPE represents pure capacitance). To obtain a reliable fitting, the additional resistive element (R_{CT}) has been added in parallel to CPE, which is assigned to the charge transfer resistance of Faradaic electrode process of oxygen reduction on gold electrode [46]. The good fitting of spectra at different stages of modification was achieved only with the complex circuit (Fig. 2S). The fitting parameters are presented in Table 1. It is seen, that the self-assembly of disulfide molecules on electrode surface led to more than 6 times decrease of electric double layer capacitance. Successive modification of electrode with nickel ions via chelating led to 18 % decrease of double layer capacitance, probably, due to the negative charge compensation within the assembled monolayer. Charge transfer resistance showed consistent decrease at different modification stages probably due to the suppression of background oxygen reduction on gold.

Affinity sorbents holding nickel or cobalt ions bind with micromolar affinity a polyhistidine terminal amino acid motif (His-tag), which is used for the recovery of His-tagged recombinant proteins in biotechnology. Recombinant firefly luciferase (N-His₆-luc) was used as a model His-tagged protein, which can easily be detected by luminescent assay. Impedance spectra showed 15 % decrease of double layer capacitance (Table 1). The experiments with protein without His-tag (bovine serum albumin) did not reveal the measurable response,

illustrating the effective protection of gold surface by SAM against nonspecific protein adsorption.

To remove N-His₆-luc from modified surface, the electrode was treated with aqueous imidazole solution. The luciferase activity was measured in washing solution. The estimated coverage of electrode surface, assessed from activity measurements, was about 1 protein molecule per 600 nm².

Atomic force microscopy

The study of nickel-modified gold surface with atomic force microscopy revealed the sufficient difference in morphology before and after modification with protein (Fig. 3). It is seen that there are spherical particles with linear dimensions of ca. 15-20 nm and height of 2-3nm. According to the previous literature data [47-50], the observed surface-attached particles might be single protein molecules adsorbed onto nickel-modified gold surface. Estimated roughness parameters are presented in Table 1S. Images are well distinguished visually. The gold grains of average diameter 100 nm are seen at blank surface. The particles of smaller dimensions with average diameter less than 20 nm assigned to adsorbed protein globules are distinguishable in protein-modified surface.

We conclude that the direct detection of recombinant protein has been carried out at nickel-modified gold surface. The reagentless manner of detection is a main attractive advantage of protocol, which can be attractive for biotechnology analysis.

Conclusions

The affinity binding between of His-tagged recombinant protein and nickel ions attached to gold surface was detected with reagentless electrochemical method. The self-assembly of disulfide molecules on gold surface was quantitatively studied by physical and electrochemical methods. The subsequent electrode modification with nickel ions and specific binding of target protein firefly luciferase with transducer have been testified in reagentless manner by impedance as a decrease of double layer capacitance and increase of charge transfer resistance assigned to background Faradaic process. The protein binding to nickel-modified electrochemical transducer has been confirmed by luciferase luminescent assay and AFM.

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University) for recombinant firefly luciferase sample. Authors thank Ilia Kabanov (Intertech corp.) and Oleg Makeev (Carl Zeiss, Russia) for AFM equipment and technical support.

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Table 1. Parameters of fitting of impedance spectra.

	R_s $k\Omega$	CPE		$C_{dl} = \left[AR_s^{-(\phi-1)} \right]^{\frac{1}{\phi}}$ nF^*	C'_{dl} $\mu F\ cm^{-2}$	R_{CT} $M\Omega$
		$A,$ $*10^{-8}$	ϕ			
Blank gold electrode	4.5	13	0.87	44.5	5.6	2.9
Gold electrode modified with disulfide	3.8	2.7	0.87	7.3	0.9	17.7
Ni-disulfide modified gold electrode	3.7	2.5	0.87	6.2	0.8	47.1
N-His ₆ -luc at Ni-disulfide modified gold electrode	3.8	2.2	0.87	5.4	0.6	63.3

*[51, 52]

Figure legends

Figure 1. Self-assembly of disulfide on gold surface. A: the chemical structure of disulfide; B: the dependence of contact angle on assembly time. Water droplets were on gold sheets treated with 1mM solution of disulfide in ethanol during different period of time. Inset: the reductive desorption of disulfide from the gold electrode surface. Solid line – electrode incubated for 2 hours in disulfide solution (point 1); dotted line – electrode after 24 hours of incubation in disulfide solution (point 2). Nitrogen-saturated 0.5M KOH, 20 mV/s.

Figure 2. Electrode modifications followed by impedance spectroscopy. The impedance spectra measured at all stages of electrode modifications are presented in complex coordinates of the frequency-normalized admittance. **A:** spectra for blank (■) and disulfide-modified electrode (□); **B:** spectra for disulfide- (□), nickel- (▲) and luciferase-modified (○) electrode; 0.1 M KCl, 0 V vs Ag/AgCl in 1M KCl, 5 mV amplitude.

Figure 3. AFM images of blank part (A) and protein modified (B) gold sheet.

Figure 1.

Figure 2.

Figure 3.