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Mendeleev Commun., 2015, **25**, 290–292
Mendeleev Communications

Direct reagentless detection of the affinity binding of recombinant His-tagged firefly luciferase with a nickel-modified gold electrode

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DOI: 10.1016/j.mencom.2015.07.021

The direct reagentless electrochemical detection of recombinant firefly luciferase binding with a gold electrode modified with nickel complex of 1,16-di[4-(2,6-dihydroxycarbonyl)pyridyl]-1,16-dioxa-8,9-dithiahexadecane has been carried out.

Immobilization onto a plane surface is a first step in the studies of protein functions and structures.[1–3] To attach the protein molecule to a metal substrate, self-assembled monolayers can be used by (i) the chemical modification of a protein with a linking structure capable of specifically reacting with the substrate to form a self-assembling monolayer (SAM)[4,5] or (ii) reactions on SAMs followed by catching the biomolecule. Site-selective methods allow one to perform the attachment of proteins with uniform orientation where the bioactive site is freely accessible for further analysis or application.[6,7] The use of bifunctional agents to bind biomaterial to solid supports is widely employed due to the ease of formation of monolayers with reasonable stability even with limited substrate diffusion towards the immobilized biological sensing element.[8]

The hexahistidine tag (His_6) is the most commonly used affinity tag for protein recovery due to its small size with a minor effect of protein native function and specific interaction with transition metal ions employing immobilized metal affinity chromatography.[9–11] The imidazole moieties of the tag-modified protein can chelate to the free coordination sites of divalent transition-metal ions which are immobilized onto solid supports *via* appropriate chelating agents such as tridentate iminodiacetic acid (IDA) or 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.

The SAMs of metal-chelating lipids[12] or thiol-functionalized chelating agents[10,11,13] 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 the kinetics and thermodynamics of specific binding between the immobilized protein and target molecules.[7,10,13(*b*),(*e*)]

In this work, the new organic ligand 1,16-di[4-(2,6-dihydroxycarbonyl)pyridyl]-1,16-dioxa-8,9-dithiahexadecane (termed disulfide hereinafter) with functional dicarboxypyridine moieties [Figure 1(*a*)] was utilized for the nickel-assisted capture of recombinant His-tagged firefly luciferase onto a polycrystalline gold electrode. The binding of luciferase with a nickel-modified SAM has been detected directly by impedance spectroscopy and confirmed by atomic force microscopy (AFM) and optical measurements of residual enzyme activity in solution for electrode surface recovery.[†]

The dimeric design of synthesized disulfide with functional moieties for the binding of divalent transition-metal ions was chosen to achieve higher complex efficiency by two adjoining functional groups and the formation of two adsorbed molecules in a single adsorption step. The modification of gold sheets surface by disulfide self-assembly was monitored by contact angle measurements with water droplets [Figure 1(*b*)]. The decrease of contact angle with self-assembly in comparison with blank gold showed surface hydrophobization due to the adsorption of disulfide molecules. The short treatment for 1 min resulted in a significant contact angle decrease owing to the fast adsorption of disulfide. Successive assembly led to the formation of a dense and uniform monolayer after 12-h self-assembly, which was utilized for the modification of gold electrodes.

Cyclic voltammetry of the ferri/ferrocyanide redox couple at the modified electrodes showed a decrease in redox currents and

[†] The experiments were carried out with Millipore (MilliQ Plus) water. All inorganic compounds were of analytical grade. Recombinant firefly luciferase N-His_6-luc (M_r 63 kDa) with a specific activity of 1.05×10^{13} relative light units per mg was provided by Dr. M. Koksharov and Professor N. Ugarova (M. V. Lomonosov Moscow State University). Contact angles were measured (±2°) with an MG horizontal microscope goniometer (Russia). For synthesis and characteristics of 1,16-di[4-(2,6-dihydroxycarbonyl)pyridyl]-1,16-dioxa-8,9-dithiahexadecane, see Online Supplementary Materials.

Voltammetric measurements were carried out using a microAutolab III electrochemical interface (Eco Chemie, Netherlands) and a three-electrode cell with separated compartments. Glassy carbon rod was used as a counter electrode. Working gold disk electrodes (diameter of 1 mm) were made from gold wire of 99.99% purity (Goodfellow, UK) pressed into a Teflon tube. The electrodes were mechanically polished with alumina powder (Al_2O_3, 0.05 μm) 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 min. Finally, the electrodes were cycled in a 0.2 M sulfuric acid in a potential range from 0 to 1.45 V at a sweep rate of 20 mV s^{-1} until a stable voltammogram. The roughness factor of the electrode, 3.9±0.2, was determined as the ratio of the true to the geometric surface area, where 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^{-1}. An Ag|AgCl electrode in 1 M KCl was used as a reference one. After pre-treatment, the electrodes were rinsed with water and 96% ethanol. Then, the electrodes were placed in a solution of disulfide for ~12 h. After that, the electrodes were rinsed with ethanol and buffer and then placed into an electrochemical cell.

Impedance measurements were performed with a Solartron 1255 Frequency Response Analyzer (Farnborough, UK) with a homemade low noise potentiostat in a two-compartment electrochemical cell. The frequency range was from 1 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.

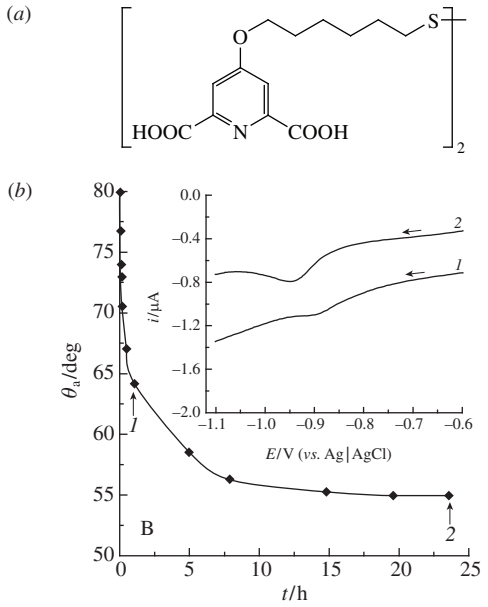

Figure 1 Self-assembly of disulfide on a gold surface: (*a*) the chemical structure of the disulfide; (*b*) contact angle *vs.* assembly time. Water droplets on gold sheets were treated with a 1 mM disulfide solution in ethanol for different times. Inset: the reductive desorption of disulfide from the gold electrode surface: (*1*) electrode incubated for 2 h in disulfide solution; (*2*) electrode after 24 h of incubation in disulfide solution. Nitrogen-saturated 0.5 M KOH, 20 mV s^{-1}.

the increase of peak-to-peak separation as compared with naked gold electrodes that indicated partial blocking of the electrode surface. The blocking properties of the layer remained during continuous cycling.

The linear sweep voltammogram recorded with a disulfide-modified gold electrode in strongly basic aqueous solution [inset in Figure 1(*b*)] presents a typical peak current of reductive desorption of disulfide molecules at –0.95 V (Ag|AgCl).[14] Charge required for desorption and calculated from the reduction peak area with one electron process assumption[15] was used for the estimation of surface concentration of adsorbed disulfide after 24-h incubation, which was 1.7 nmol cm^{-2}. Taking into account the roughness coefficient of the gold electrodes of about 3.9,[15,16] the value of 0.4 nmol cm^{-2} for disulfide surface concentration was calculated, which is close to a theoretical value of 0.8 nmol cm^{-2} for a condensed monolayer.[14(a)] Lower than theoretical density can be explained by Coulomb repulsion between the negatively charged carboxy groups of the disulfide and larger molecule dimensions. LSV recorded with electrodes modified with non-dense SAM (1.5-h assembly) showed a smaller peak of reductive desorption corresponding to a 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. Figure 2 represents the impedance spectra in complex coordinates of the frequency-normalized admittance. The diameter of semicircle observed in all spectra corresponds to the double layer capacitance at the electrode–solution interface. The disulfide self-assembly [Figure 2(*a*)] caused a decrease of the double layer capacitance by a factor of 5 due to the extension of the effective thickness of capacitor of the electric double layer and decreased dielectric permittivity of the interface layer.[17] Subsequent electrode modifications with nickel ions and protein led to less prominent decreases in double layer capacitance [Figure 2(*b*)] owing to the removal of binding from the actual double layer region, which dominates the double layer capacitance.

To quantitatively assess changes in electrode interfacial properties due to successive modifications, the equivalent circuit analysis was carried out. Taking into account the roughness of the electrode surface, the constant phase element (CPE) was used instead of pure capacitance. CPE is represented by two fitting parameters: A and φ, which varied from 0 to 1 (when φ is 0, the CPE behaves as a pure resistance; when $\varphi = 1$, the CPE represents pure capacitance). To obtain a reliable fitting, the additional resistive element (R_{CT}) was added in parallel to CPE, which is assigned to the charge transfer resistance of Faradaic electrode process of oxygen reduction on gold electrode.[18] The good fitting of spectra at different stages of modification was achieved only with the complex circuit (Table 1). The self-assembly of disulfide molecules on electrode surface led to a decrease in the electric double layer capacitance by a factor of >6. Successive modification of electrode with nickel ions *via* chelating led to 18% decrease in double layer capacitance,

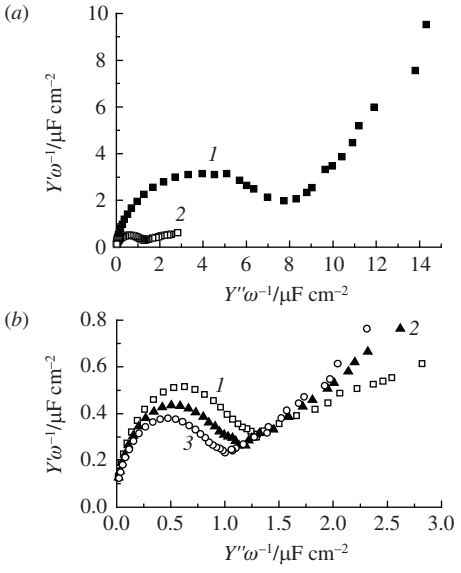

Figure 2 Electrode modifications followed by impedance spectroscopy: (*a*) the spectra of (*1*) blank and (*2*) disulfide-modified electrode; (*b*) the spectra of (*1*) disulfide-, (*2*) nickel- and (*3*) luciferase-modified electrodes; 0.1 M KCl; 0 V *vs.* Ag/AgCl in 1 M KCl; 5 mV amplitude.

Silica sheets (5×5×0.1 mm) with sputtered gold (thickness of 50±10 nm) were purchased from NT-MDT (Russia). The sheets were annealed by gas-air flame for 5 s and cooled under argon stream. Then, the sheets were placed in a 10 mM 1,16-di[4-(2,6-dihydroxycarbonyl)pyridyl]-1,16-dioxa-8,9-dithiahexadecane 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 h. After washing with ultrapure water, the modified sheet was placed in 1 μM N-His_6-luc solution in a 0.1 M KCl, 0.01 M phosphate buffer at pH 7 for 2 h. Finally, the sheet was carefully washed with a large volume of the buffer and ultrapure water. The prepared sheets were carefully blown with argon and kept for several hours in a Petri dish at room temperature before AFM measurements with a MultiMode V atomic force microscope (Veeco, USA). The modified and unmodified parts of the plate were scanned at least in three different points using tapping mode with a cantilever frequency of 314.7 kHz. A silicon cantilever with constant of resilience 0.03 N m^{-1} (Ultrasharp CSG, NT-MDT, Russia) was used as a probe. The recorded images (1×1 μm) were corrected by fitting and subtracting of 3rd order polynomial, and then roughness parameters were calculated for two different sections of each picture with areas of about 270 and 135 nm^2 using the NanoScope 7.2 software.

The N-His_6-luc activity was measured with an FB12 luminometer (Zylix Corp., USA) by the maximum of light intensity emitted due to the enzymatic reaction in saturated substrates concentrations. An aliquot portion (5 μl) of an enzyme solution was added to 350 μl of a 1.7 mM solution of ATP in a 50 mM TRIS-acetate buffer solution (2 mM EDTA, 10 mM $MgSO_4$, pH 7.8).

Table 1 Parameters of fitting of impedance spectra.

Electrode	R_S/ kΩ	CPE		$C_{dl} = [AR_S^{-(\phi-1)}]^{1/\phi}$/ nF	C'_{dl}/ μF cm^{-2}	R_{CT}/ MΩ
		A	φ			
Blank gold electrode	4.5	13×10^{-8}	0.87	44.5	5.6	2.9
Gold electrode modified with disulfide	3.8	2.7×10^{-8}	0.87	7.3	0.9	17.7
Ni–disulfide-modified gold electrode	3.7	2.5×10^{-8}	0.87	6.2	0.8	47.1
N-His_6-luc at Ni–disulfide-modified gold electrode	3.8	2.2×10^{-8}	0.87	5.4	0.6	63.3

probably, due to the negative charge compensation within the assembled monolayer. Charge transfer resistance showed consistent decrease at different modification stages, apparently, owing to the suppression of background oxygen reduction on gold.

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-taged recombinant proteins in biotechnology. Recombinant firefly luciferase (N-His_6-luc) was used as a model His-tagged protein, which can be easily detected by a luminescent assay. Impedance spectra exhibited a 15% decrease in 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.

Based on the CVA data, the formed layer is stable in a potential range of –0.9 to +0.9 V. To remove N-His_6-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 nm^2.

The study of nickel-modified gold surface by AFM revealed a sufficient difference in the morphology before and after modification with protein (Figure 3). There are spherical particles with linear dimensions of 15–20 nm and a height of 2–3 nm. According to previous data,[19] the observed surface-attached particles might be single protein molecules adsorbed onto a nickel-modified gold surface. The gold grains of an average diameter of 100 nm can be seen at a blank surface. The particles with an average diameter of <20 nm assigned to adsorbed protein globules are distinguishable in protein-modified surface.

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

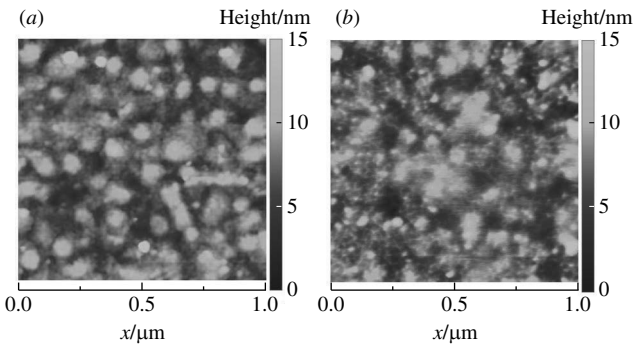

Figure 3 AFM images of (*a*) a blank part and (*b*) a protein-modified gold sheet.

This work was supported by the Russian Foundation for Basic Research (grant no. 09-03-00775-a). We are grateful to Dr. M. I. Koksharov (M. V. Lomonosov Moscow State University) for recombinant firefly luciferase sample and to I. Kabanov (Intertech Corp.) and O. Makeev (Carl Zeiss, Russia) for AFM equipment and technical support.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2015.07.021.

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Received: 12th September 2014; Com. 14/4464