Electronic Supplementary Material (ESM)
This journal is © The Royal Society of Chemistry 2022

Electrochemically Stimulated Protein Release from pH-Switchable Electrode-Immobilized Nitroavidin-Biotin and Avidin-Iminobiotin Systems

Ronaldo Badenhorst, Evgeny Katz,* Oleh Smutok*

Department of Chemistry and Biomolecular Science, Clarkson University, Potsdam, NY 13699-5810, USA

* Corresponding authors; emails: osmutok@clarkson.edu; ekatz@clarkson.edu

Electronic Supplementary Material

Chemicals and materials

Avidin (from egg white; BioUltra, lyophilized powder, ≥10 units/mg protein (E1%/280), ≥98%; SDS-PAGE), biotin (98%), and 2-iminobiotin (≥98%; TLC) were purchased from Acros Organics. 1-Pyrenebutyric acid *N*-hydroxysuccinimide ester (PBSE; 95%), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES buffer; ≥99.5%), biotin *N*-hydroxysuccinimide ester, tetranitromethane, sodium ascorbate, and other standard organic and inorganic materials, solvents, and reagents were purchased from MilliporeSigma (formerly Sigma-Aldrich). All commercial reagents were used as supplied without further purification. Nitro-avidine was synthesized by using the procedure reported earlier.[1] The biotin-FITC derivative and iminobiotin-active ester derivative were synthesized and characterized according to the recently published report.[2] Structures of biotin, iminobiotin and their active ester derivatives are shown in Figure ESM1. Buckypaper composed of compressed multiwalled carbon nanotubes (MWCNTs; Buckeye Composites, NanoTechLabs, Yadkinville, NC) was used as the electrode material (geometric area ca. 0.25 cm^2). All experiments were carried out in ultrapure water (18.2 MΩ·cm; Barnstead NANOpure Diamond) at room temperature (22 $\pm$ 2 °C).

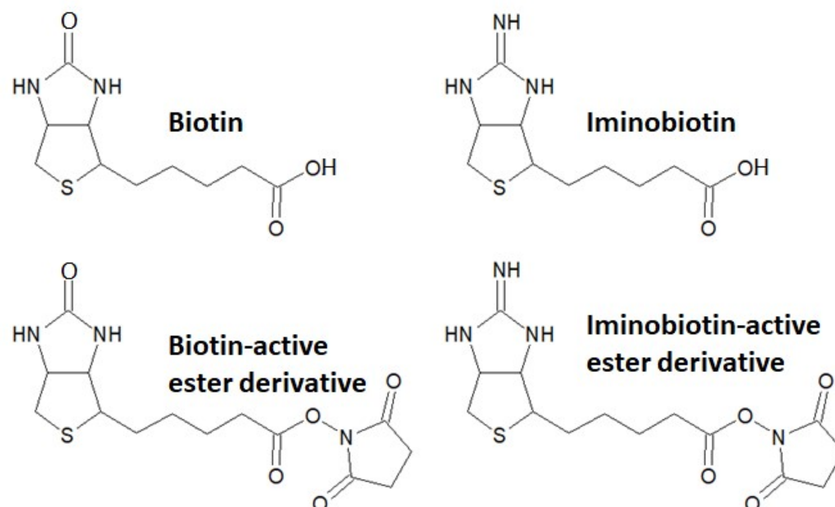

Figure ESM1. Structures of biotin and iminobiotin and their active ester derivatives.

Instrumentation

Electrochemical experiments were conducted using an electrochemical workstation (ECO Chemie Autolab PASTAT 10) and GPES 4.9 (General Purpose Electrochemical System) software. While performing electrochemical experiments (cyclic voltammetry and constant potential electrolysis) the potentials were measured using a BASi Ag|AgCl|KCl, 3 M, reference electrode, and a graphite slab was used as a counter electrode. Fluorescence measurements were performed using a fluorescent spectrophotometer (Varian, Cary Eclipse). Fluorescent images were obtained with Leica TCS SP5 II Tandem Scanning Confocal and Multiphoton Microscope. ImageJ software was used for quantifying fluorescence intensity of the images. pH values of the solutions were adjusted using a Mettler Toledo S20 SevenEasy pH meter. High Performance Liquid Chromatography (HPLC) was performed using an Agilent/HP Ti 1050 Series System with a Phenomenex Luna 5u C18 100A column with 150×4.60 mm dimensions and an internal diameter of 5 micron.

Modification of the electrodes with avidin or nitro-avidin

Buckypaper pieces (1 cm^2 total geometrical area; 0.25 cm^2 electrochemically active geometrical area immersed in the electrolyte solution during measurements) were rinsed with isopropanol for 15 min and left on filter paper to dry for 5 min. Then, the buckypaper was immersed in 10 mM 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBSE) solution in dimethyl sulfoxide (DMSO) under moderate shaking for 1 h. Then, the electrodes were rinsed with DMSO for removing excess of PBSE, followed by rinsing with 3 mM HEPES buffer, pH 8.5, for removing DMSO. The modified electrodes were cast with 10 µL of (nitro)avidin (5 mg/mL in HEPES buffer, pH 8.5) and left for 1 h in the dark with 100 % humidity.

Binding of biotin/iminobiotin active ester derivatives to the nitroavidin/avidin-functionalized electrodes

The buckypaper electrodes modified with covalently immobilized avidin or nitroavidin were reacted with iminobiotin or biotin active ester derivatives (0.5 mM in 3 mM of HEPES buffer, pH 7.0), respectively. The reaction proceeded for 1 hr in the dark with 100% humidity. In the majority of the experiments, the reaction solution included iminobiotin or biotin missing the active ester groups applied at the ratio 1:1 to the iminobiotin or biotin with the active ester groups. Further, the electrodes were rinsed with 3 mM HEPES buffer, pH 7.0, three times for 2-3 minutes on an orbital shaker at medium shaking intensity.

Possible side reactions

The bifunctional linker, composed of biotin (or iminobiotin) and an active ester group, can react with the surface confined nitroavidin (or avidin) in two different ways. One of the options is the formation of the affinity complexes nitroavidin/biotin (or avidin/iminobiotin). In this case the active ester group remains free and available for immobilization of BSA. This is the desired reaction illustrated in Figure 1 in the paper. A side reaction may proceed between the active ester groups and amino groups of lysine residues in the avidin or nitroavidin backbone. It should be noted that avidin and nitroavidin have only 3 lysine amino acids in the protein structure (Lys-45, Lys-94, Lys-111).[3] Some of them (possibly all of them) are blocked upon immobilization of avidin or nitroavidin at the buckypaper electrode (Figure 1 in the paper), therefore they cannot react with the active ester groups of the biotin or iminobiotin derivatives. If anyway, some of the lysine residuals react with the active ester groups, this reaction does not offer active ester groups for immobilization of BSA, therefore this side reaction does not interfere with the formation of the avidin/nitroavidin and BSA structures.

BSA fluorescent labeling with Rhodamine-B (RhD-B)

Rhodamine B isothiocyanate was dissolved in a carbonate/bi-carbonate buffer (CBB; 50 mM, pH 9.0) with a final concentration of 1 mg/mL. The formed RhD-B solution (200 mL) was added dropwise to a 1 mL BSA solution (3 mg/mL in 50 mM CBB, pH 9.0). The mixture was stirred for 12 h in the dark at 4 °C. The product was purified by size-exclusion chromatography with Sephadex-50 in 25 mM HEPES buffer, pH 7.4. The first fraction was collected, and the solvent was removed by freeze-drying. The final product (BSA-RhD-B) was dissolved in 25 mM HEPES buffer, pH 7.4 with a final concentration of 1 mg/mL used for electrode casting.

Immobilization of BSA-RhD-B onto the (imino)biotin-functionalized electrodes

The biotin- / iminobiotin-functionalized electrodes were reacted with 10 µL of BSA-RhD-B (1 mg/mL in 25 mM of HEPES buffer, pH 7.4) and left for 2 hours in the dark with 100% humidity.

Then, the electrodes were rinsed with 3 mM HEPES buffer, pH 7.0, five times for 2-3 minutes on an orbital shaker at medium shaking intensity. Once washing was complete, the electrodes were used in further experiments.

Electrochemically stimulated BSA-RhD-B release

A potential of -0.4 V (vs. Ag/AgCl) was applied on the electrode modified with nitroavidin/biotin-BSA-RhD-B. Electrochemical O_2 reduction (see cyclic voltammogram (b) in Figure ESM2A) resulted in consumption of H^+ ions, thus resulting in pH increase, dissociation of the affinity complex of nitroavidin/biotin, and release of BSA-RhD-B. Note that the potentials more negative than -0.4 V were not applied to avoid electrochemical decomposition of the RhD-B fluorescent label.

A potential of +0.2 V (vs. Ag/AgCl) was applied on the electrode modified with avidin/iminobiotin-BSA-RhD-B. Electrochemical ascorbate (1 mM) oxidation (see cyclic voltammogram (b) in Figure ESM2B) resulted in release of H^+ ions, thus resulting in pH decrease, dissociation of the affinity complex of avidin/iminobiotin, and release of BSA-RhD-B.

Note that the electrochemically stimulated pH changes resulting in the BSA-RhD-B proceed in 3 mM HEPES buffer containing 0.1 M Na_2SO_4 (pH 7.0 for O_2 reduction and pH 8.5 for ascorbate oxidation). The used buffer capacity allowed local (interfacial) pH changes upon performing electrochemical reactions, but it was preventing bulk pH changes.

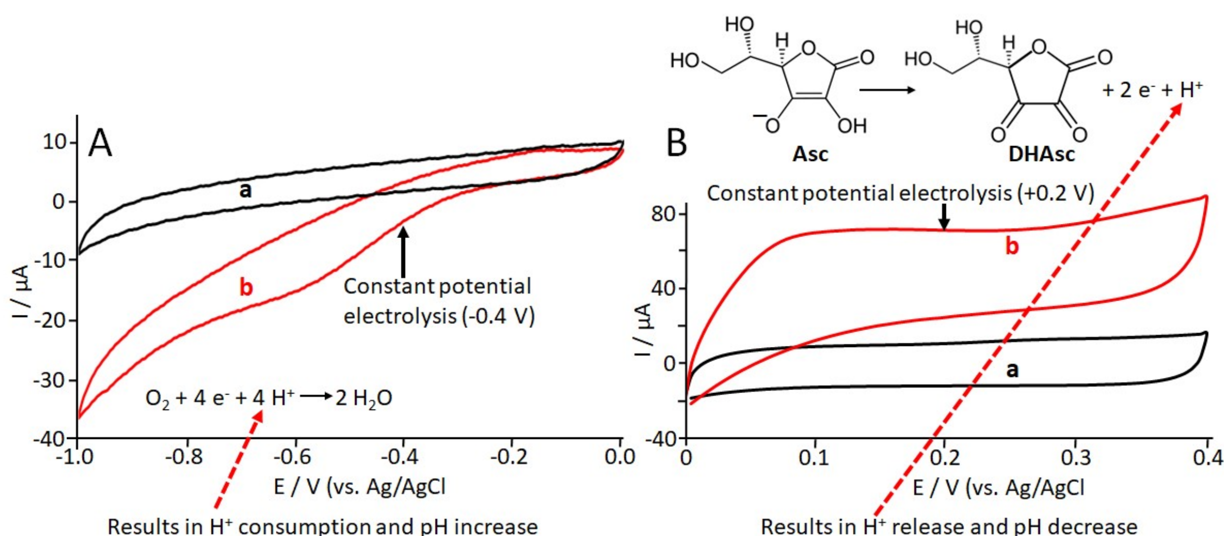

Figure ESM2. Cyclic voltammograms obtained with a buckypaper electrode modified with nitroavidin (A): (a) in the absence of O_2 (under Ar), (b) in the presence of O_2 (in equilibrium with air) and another electrode modified with avidin (B): (a) in the absence (a) and (b) presence of ascorbate (1 mM). The background electrolyte was composed of 3 mM HEPES buffer, pH 7.0 for O_2 reduction and pH 8.5 for ascorbate oxidation, containing 0.1 M Na_2SO_4. Potential scan rate, 10 mV/s.

Immobilization stability dependence on the number of chemical bonds

It should be noted that multipoint covalent immobilization of proteins is always more stable than a similar single covalent immobilization. A good reference example might be protein immobilization with glutaric dialdehyde. The reaction of amino groups in a protein backbone (in lysine residuals) with an aldehyde group results in an unstable Schiff base bond. Therefore, glutaric dialdehyde cannot be a stable linker for immobilization of small organic molecules having only one amino group. To solve this problem, the produced Schiff base bond needs to be reduced with $NaBH_4$ for formation of a stable covalent bond. However, this is not needed in the case of immobilization of proteins forming several Schiff base bonds. While each of them is unstable, their cooperative effect results in very stable protein immobilization.[4-6] Overall, this example demonstrates the cooperative effect of many unstable chemical bonds that stabilize immobilization of proteins.
Related to the system reported in the present paper, we observed strong dependence of the number of bonds on stability of the protein (BSA) immobilization. Reducing the number of connecting bonds resulted in easier release of the immobilized BSA when the stability of the affinity bonds was decreased with pH changes. This experimentally observed phenomenon explained verbally can be formulated with mathematical expressions.
Let's assume that BSA is bound to the avidin/iminobiotin or nitroavidin/biotin complexes with *n* bonds, where *n* is represented with bonds numbered 1, 2, …… *i* …*n*. If the binding is reversible, the dissociation constant K_{di} for the *i* bond can be expressed according to Eq. 1, where S_{di} and S_{bi} are surface concentration of BSA with dissociated and bound *i* bonds:

$$K_{di} = \frac{S_{di}}{S_{bi}} \quad \text{(Eq. 1)}$$

The system state with all *n* bonds dissociated (corresponding to the protein release) can be expressed with Eq. 2:

$$\sum_{i=1}^{n} S_{di} = \sum_{i=1}^{n} \left(K_{di} \cdot S_{bi}\right) = K_d \sum_{i=1}^{n} S_{bi} = K_d \cdot nS_b \quad \text{(Eq. 2)}$$

This mathematical expression requires two assumptions: (a) all dissociation constant for all *n* bonds are equal, then K_{di} can be changed to K_d and placed outside the sum operator; (b) all surface concentrations of the bound chemical bonds are equal, then the sum operation can be changed to product nS_b.

Overall, the total dissociated state of the system depends on the dissociation constant of each single bond (K_d), which is pH-dependent, and on the number of bonds (*n*). Equation 2 allows to conclude that the increase of the number of bonds results in decrease of the total dissociation (all bonds dissociated).

References

1 M. Wilchek, E. A. Bayer, Avidin-biotin immobilization systems, in: *Immobilized Biomolecules in Analysis: A Practical Approach*, T. Cass, F. S. Ligler (Eds.), Oxford University Press, 1999, pp. 15–35.

2 R. Badenhorst, V. Krishna Kadambar, M. Bellare, A. Melman, E. Katz, O. Smutok, *Phys. Chem. Chem. Phys.* 2022, **24**, 6410–6414.

3 G. Gitlin, E. A. Bayer, M. Wilchek, *Biochem. J.* 1987, **242**, 923–926.

4 Y. Bai, C. G. Koh, M. Boreman, Y.-J. Juang, I.-C. Tang, L. J. Lee, S.-T. Yang, *Langmuir* 2006, **22**, 9458–9467.

5 H. H. Weetall, *Anal. Chem.* 1974, **46**, 602A– 604A.

6 A. Rembaum, S. Margel, J. Levy, *J. Immunological Meth.* 1978, **2**, 239—250.