Electronic Supplementary Material (ESM)

Electrochemically Produced Local pH Changes Stimulating (Bio)Molecule Release from pH-Switchable Electrode-Immobilized Avidin-Biotin Systems

Ronaldo Badenhorst,[‡] Vasantha Krishna Kadambar,[‡] Madhura Bellare, Artem Melman,[&] 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
[‡] These authors contributed equally.
[&] Deceased

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. Microperoxidase-11 (MP-11; ≥85%; HPLC), 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBSE; 95%), *N*-hydroxysuccinimide (NHS; 98%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; >98%), *N*,*N*'-disuccinimidyl carbonate (>95%), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES buffer; ≥99.5%), trifluoroacetic acid (TFA; >99%), 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] All experiments were carried out in ultrapure water (18.2 MΩ·cm; Barnstead NANOpure Diamond) at room temperature (22 ± 2 °C). 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).

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. SpectraMax i3x Multi-Mode Microplate Reader + MiniMax 300 Imaging Cytometer (Molecular Devices) was used for the analysis of the release of the biotinylated MP-11. Fluorescence measurements were performed using a fluorescent spectrophotometer (Varian, Cary Eclipse). A Shimadzu UV-2450 UV–Vis spectrophotometer and an automatic microplate absorbance reader were used for optical absorbance measurements.

pH values of the solutions were adjusted using a Mettler Toledo S20 SevenEasy pH meter. Mass spectra were recorded in Waters Micromass Q-Tof mass spectrometer.

Synthesis of the biotin–fluorescein conjugate.

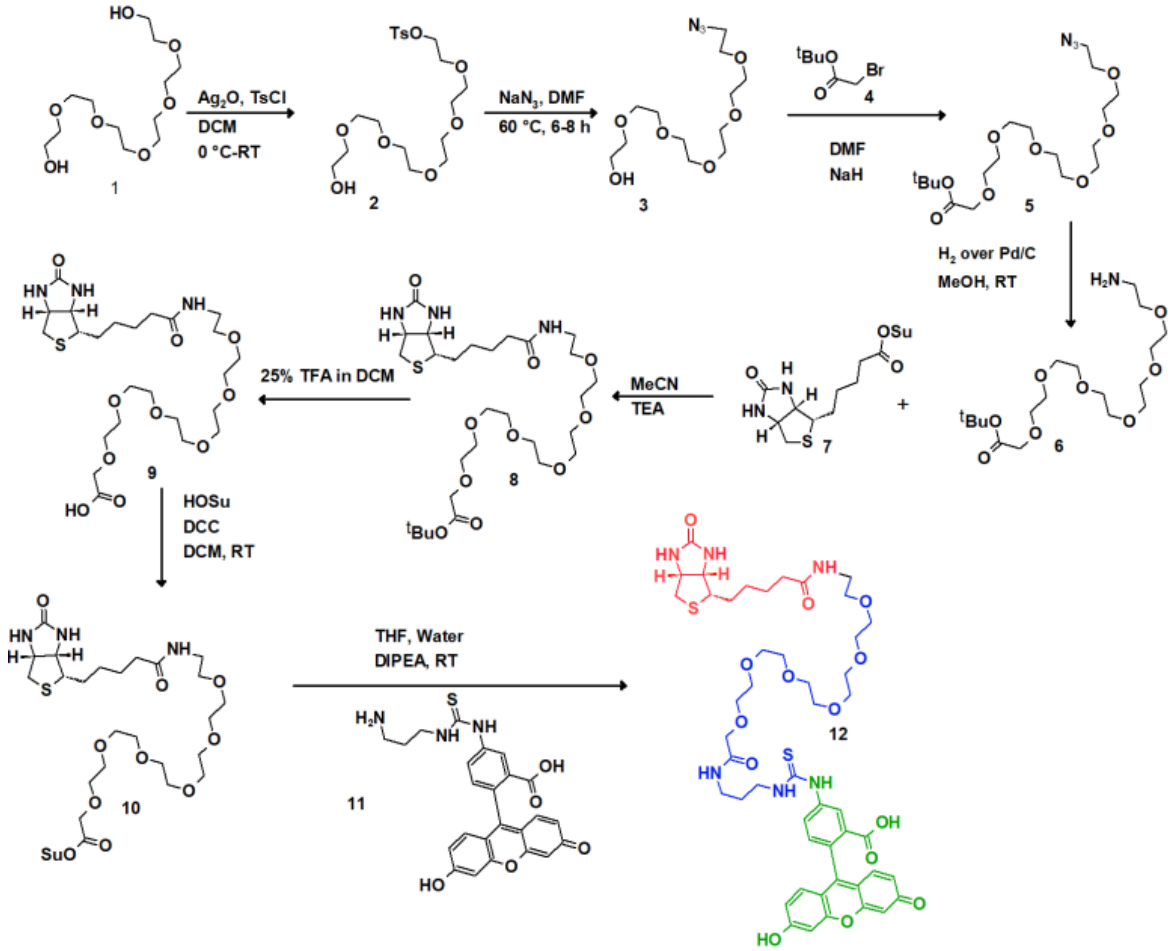


Figure ESM1. Synthetic steps in preparation of biotin–fluorescein conjugate.

The stepwise preparation of the biotin – fluorescein conjugate:

tert-Butyl-2-[amino(hexaethyleneoxy)]acetate **6** was synthesized according to the procedure reported earlier.[2] (Abbreviations used in the first reaction steps: DCM – dichloromethane, TsCl – 4-toluenesulfonyl chloride, DMF – dimethylformamide, MeOH – methanol, RT – room temperature.) Biotin-NHS ester **7** was synthesized as per the literature procedure reported elsewhere.[3] Trimethylamine (TEA) (140 mg, 1.3 mmol) was added to a solution of *tert*-butyl 2-[amino(hexaethyleneoxy)]acetate **6** (500 mg, 1.2 mmol) and biotin-NHS ester **7** (474 mg, 1.3 mmol) in acetonitrile (MeCN) (5 mL) and stirred for 3 hours at room temperature (RT) (22 ± 2 °C). After the reaction, the solvent was evaporated from the reaction mixture, which was then purified by silica gel column chromatography (5-10% MeOH in $CHCl_3$) to yield biotin-hexaethylene glycol derivative (biotin-HEG), **8** (550 mg, 70%). The resultant product was dissolved in

dichloromethane (DCM) containing 25% v/v trifluoroacetic acid (TFA) (5 mL) and the solution was stirred at room temperature overnight. Then, the solvent was evaporated under reduced pressure to yield biotin-HEG-carboxylic acid **9** (500 mg, 100%). The obtained biotin-HEG-carboxylic acid **9** (100 mg, 0.18 mmol) and hydroxysuccinimide (HOSu) (20 mg, 0.18 mmol) were dissolved in dichloromethane (1 mL) and *N*,*N*'-dicyclohexylcarbodiimide (DCC) (37 mg, 0.18 mmol) in dichloromethane (DCM) (0.5 mL) was added to the solution under stirring. The reaction mixture was stirred at RT for 3 h, filtered to remove dicyclohexyl urea (DCU) by-product and the solvent was evaporated under reduced pressure to obtain the NHS ester **10** (117 mg, 100%). It was used in the next reaction step without further purification. The NHS ester **10** (117 mg, 0.18 mmol) was dissolved in tetrahydrofuran (THF) (1 mL). A solution of FITC amine[4] **11** (81 mg, 0.18 mmol) and *N*,*N*'-diisopropylethylamine (DIPEA) (68 mg, 0.54 mmol) in water (1 mL) was added to the mixture produced at the previous step under RT. The resultant reaction mixture was stirred at RT for 3 h. Then, the solvent was evaporated under reduced pressure and the product was purified by silica gel column chromatography (20% MeOH in $CHCl_3$) to yield the final product **12** (89 mg, 50%). ^{1}H NMR (400 MHz, chloroform-d) δ 1.36 - 1.50 (m, 2 H), 1.56 - 1.79 (m, 4 H), 1.90 (m, 2 H), 2.15 - 2.30 (m, 2 H), 2.68 - 2.79 (m, 1 H), 2.86 - 2.98 (m, 2 H), 3.02 (d, *J* = 3.27 Hz, 1 H), 3.15 - 3.26 (m, 1 H), 3.37 (br. s., 3 H), 3.55 (d, *J* = 4.03 Hz, 2 H), 3.59 - 3.79 (m, 18 H), 4.03 (d, *J* = 3.53 Hz, 2 H), 4.31 (d, *J* = 4.03 Hz, 1 H), 4.50 (br. s., 1 H), 6.57 (d, *J* = 8.81 Hz, 2 H), 6.68 - 6.77 (m, 3 H), 7.20 (dd, *J* = 8.18, 3.90 Hz, 1 H), 7.80 (br. s., 1 H), 7.90 - 8.04 (m, 1 H), 8.16 (br. s., 3 H) ^{13}C NMR (101 MHz, CHLOROFORM-d) δ 25.4, 28.1, 28.3, 28.7, 35.4, 35.6, 35.8, 39.0, 39.7, 41.4, 46.6, 47.0, 47.2, 47.4, 47.6, 47.8, 48.0, 48.2, 48.5, 55.6, 60.2, 62.0, 69.2, 69.8, 70.0, 70.1, 70.7, 102.2, 110.1, 112.3, 124.4, 127.7, 128.8, 129.0, 152.8, 160.1, 164.7, 169.7, 171.6, 174.7. $[M+Na]^+$ = 1033.3657 Da Found = 1033.3647 Da.

Characterization of the synthesized product 12.

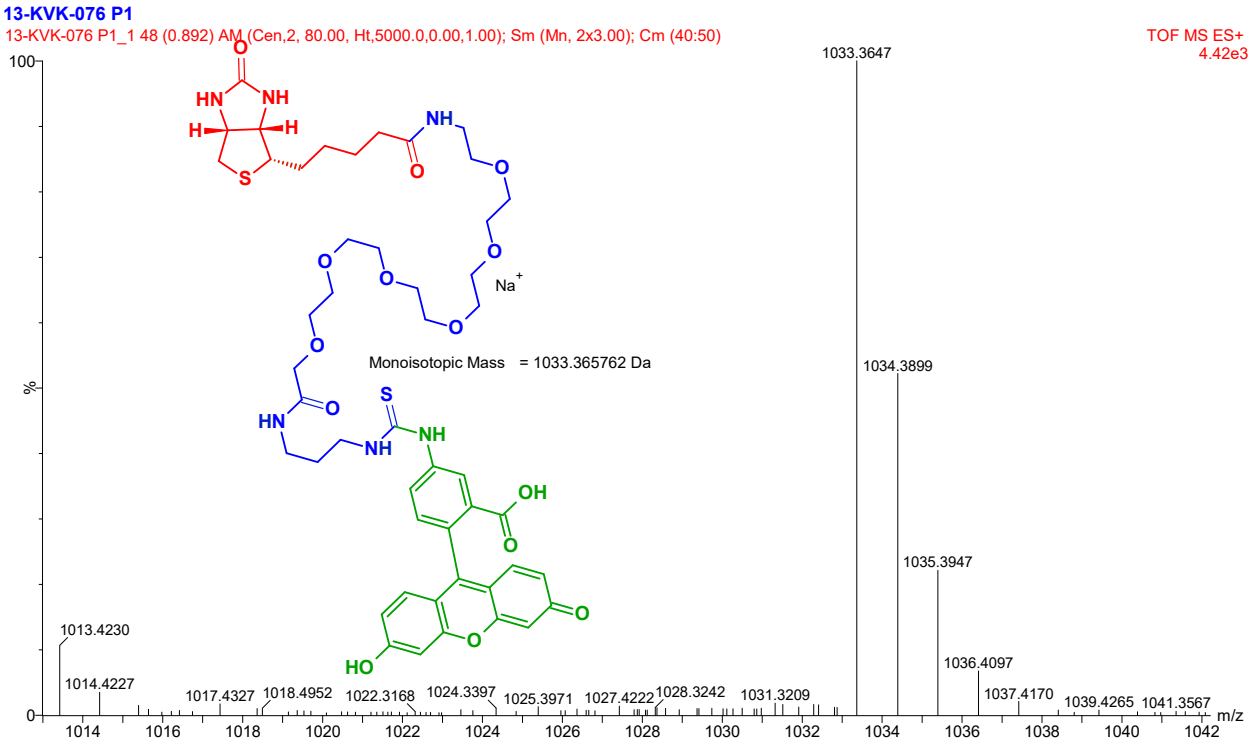


Figure ESM2. Mass spectrum of **12**.

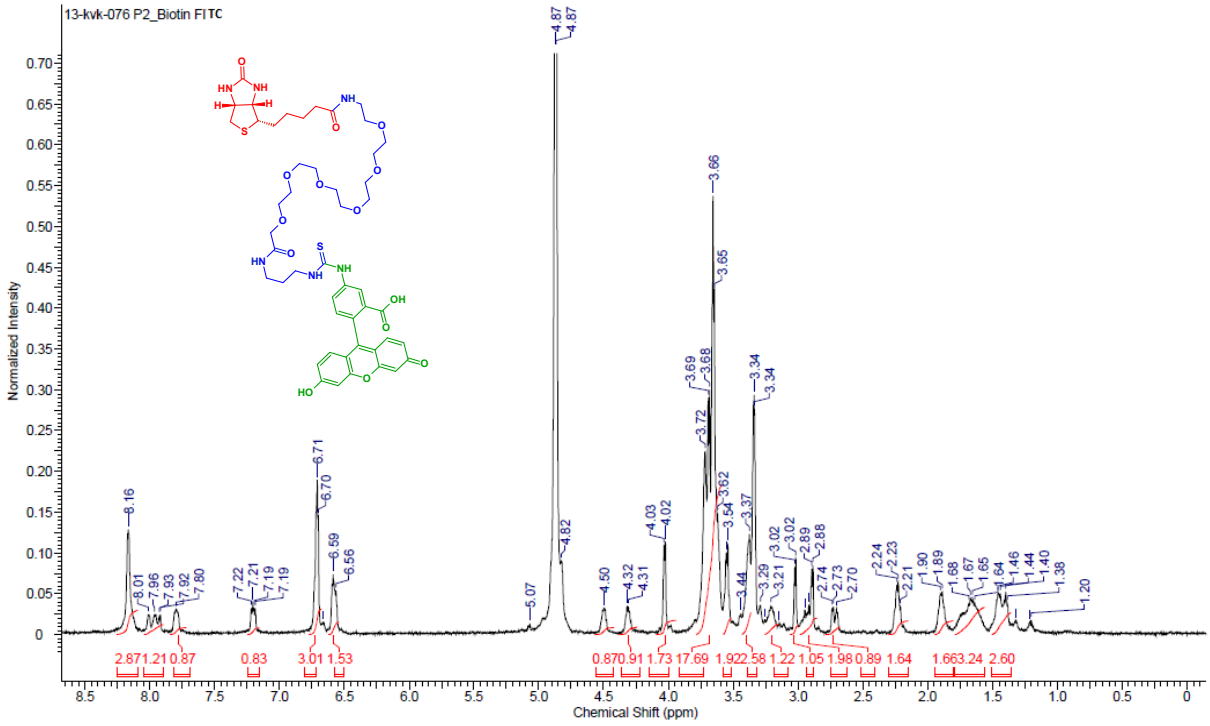


Figure ESM3. ^{1}H NMR spectrum of **12**.

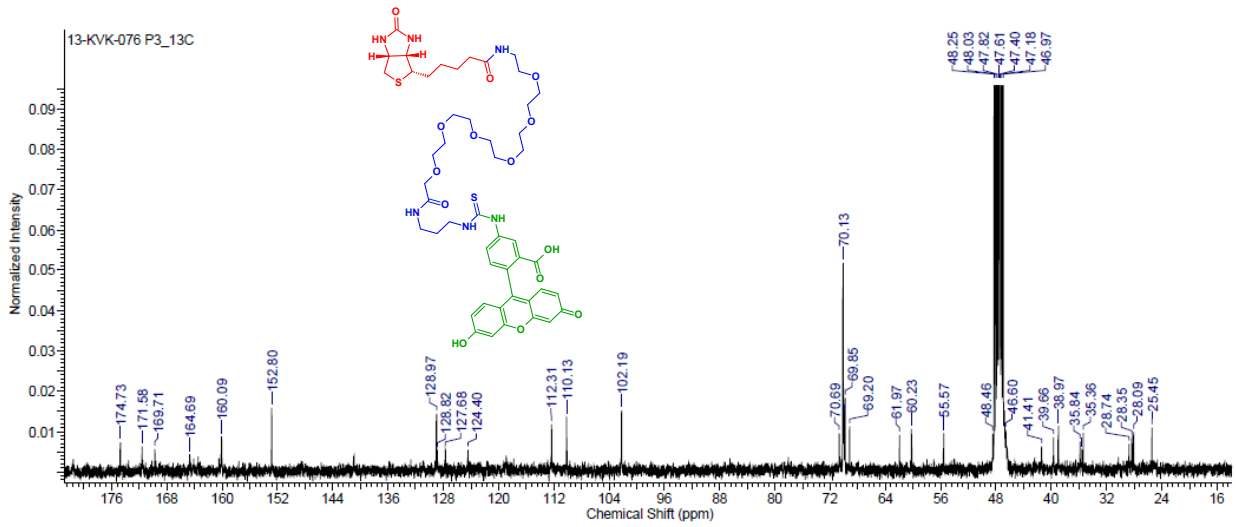


Figure ESM4. ^{13}C NMR spectrum of **12**.

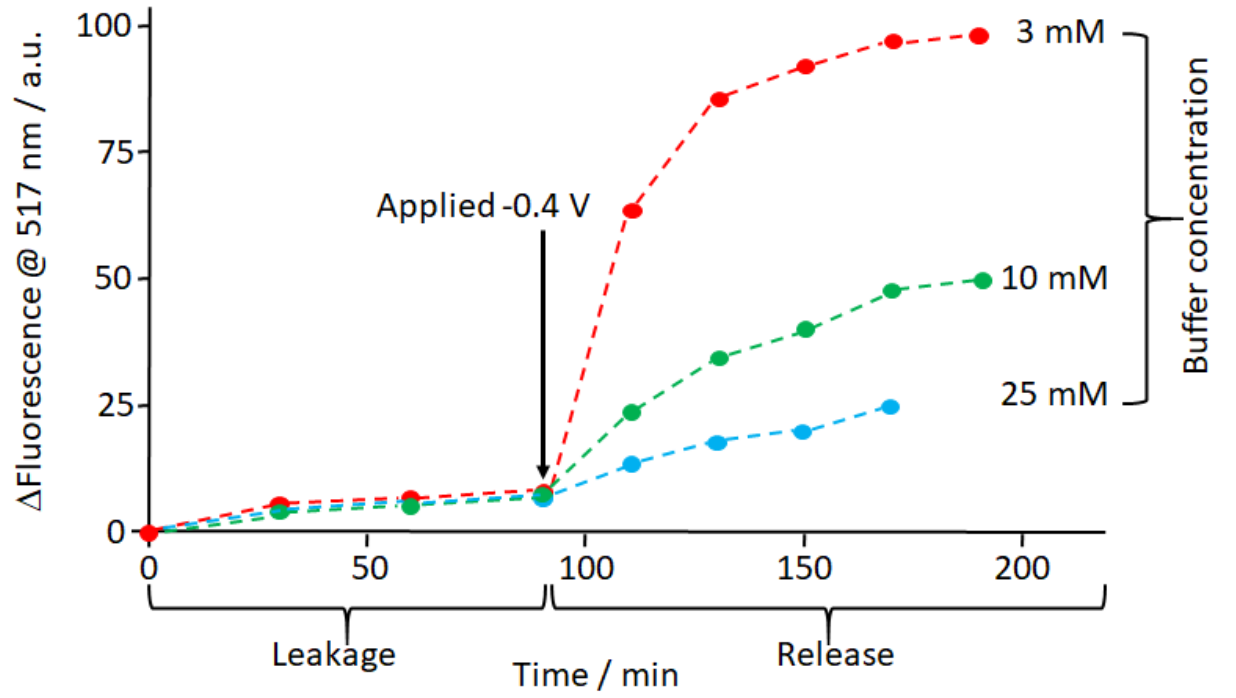


Figure ESM5. Electrochemically stimulated release of the biotinylated dye from the nitro-avidin-modified buckypaper electrode upon application of -0.4 V potential (vs. Ag/AgCl). The background solution was composed of a HEPES buffer (with various concentrations: 3 mM, 10 mM, 25 mM), pH 7.0, containing 0.1 M Na_2SO_4 and O_2 in equilibrium with air. Note that the increased buffer concentration inhibits the release process.

Comment to the experimental conditions used in the electrochemical released of the fluorescent dye:

It should be noted that we were limited by the redox stability of the immobilized fluorescent dye, which is getting decomposed when the applied reductive potential is above -0.5 V (vs. Ag/AgCl reference electrode). Therefore, we applied the minimum required potential (-0.4 V) needed for the O_2 reduction. This limitation may not apply to other molecule-releasing systems with more redox-stable species.

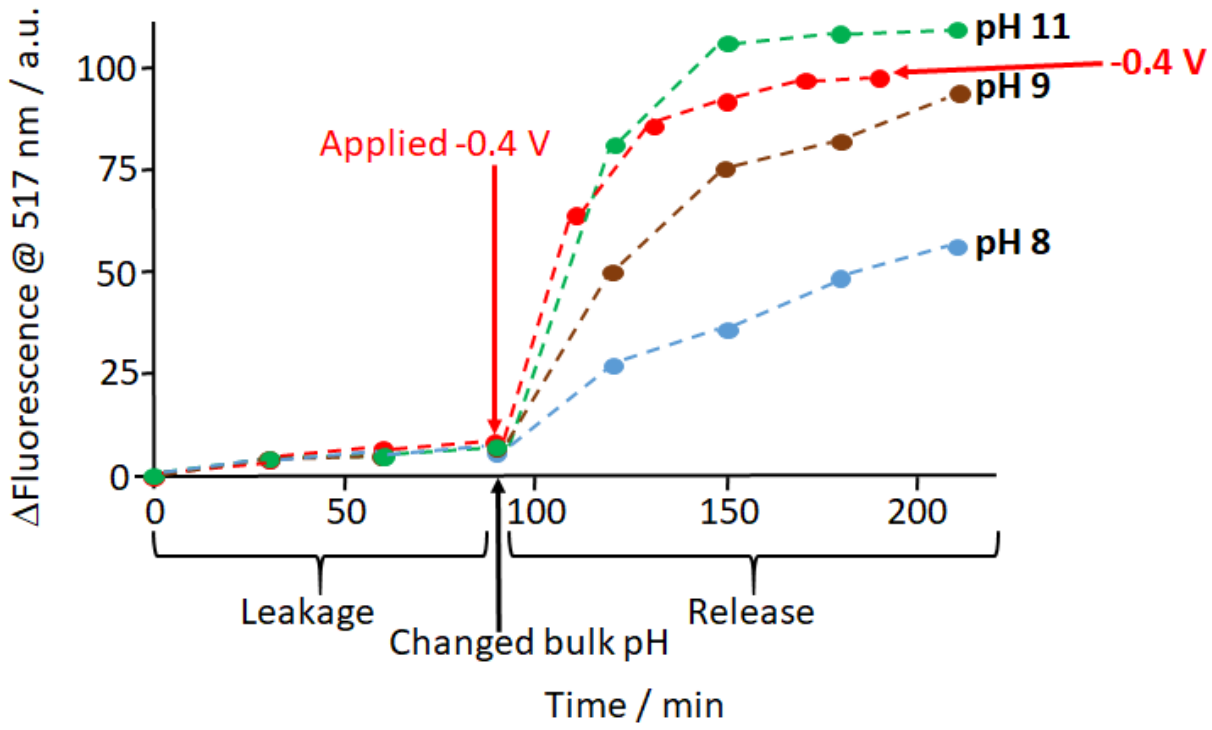


Figure ESM6. Electrochemically stimulated release of the biotinylated dye from the nitro-avidin-modified buckypaper electrode upon application of -0.4 V potential (vs. Ag/AgCl) compared with the release to buffer solutions with different bulk pH values (pH 8, 9, 11). The solutions with the various pH values included 50 mM HEPES buffer with added 0.1 M Na_2SO_4. The solution for the electrochemically stimulated release was composed of 3 mM HEPES buffer, pH 7.0, with added 0.1 M Na_2SO_4. The measurements were performed under air.

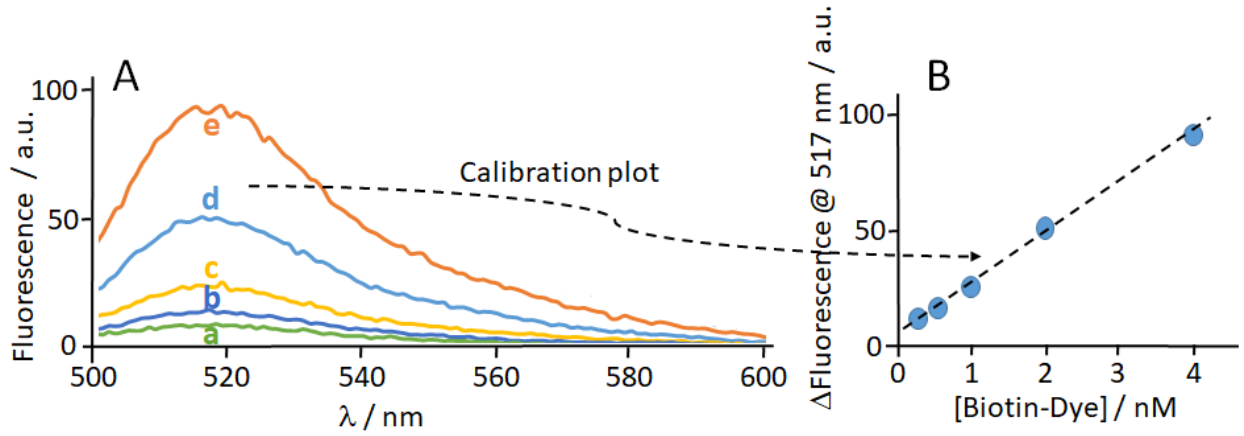


Figure ESM7. (A) The fluorescence spectra obtained for biotinylated fluorescein derivative with different concentrations: (a) 0.25 nM, (b) 0.5 nM, (c) 1 nM, (d) 2 nM, (e) 4 nM. The background solution was composed of a 3 mM HEPES buffer, pH 7.0, containing 0.1 M Na_2SO_4 and O_2 in equilibrium with air. (B) The calibration plot derived from the spectra in (A).

Synthesis of iminobiotin succinimide ester

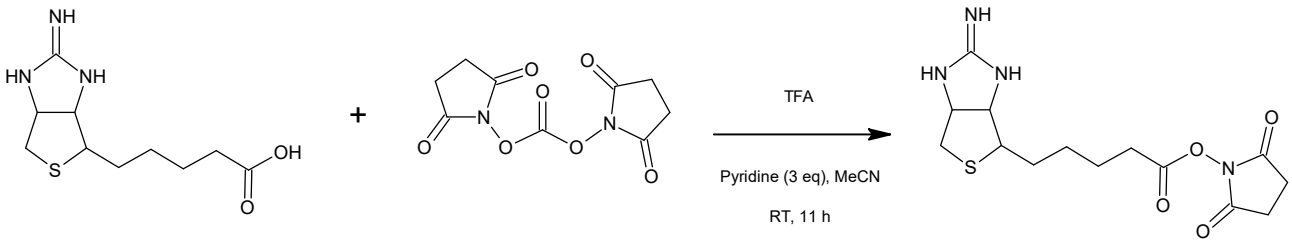


Figure ESM8. The scheme of synthesis of iminobiotin succinimide ester.

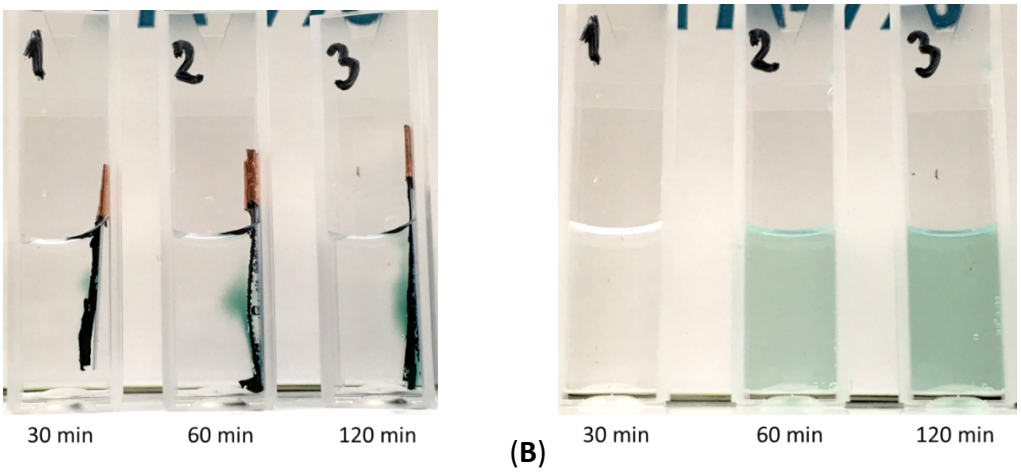
The syntheis was performed according to the published procedure.[5] Excess of trifluoroacetic acid (TFA) was added to 2-iminobiotin (88.0 mg, 0.36 mmol) in an acetonitrile solution and the resulting mixture was stirred for 2 h. The excessive TFA was then evaporated off under reduced pressure. A solution of the resulting product, pyridine (82.5 µL, 0.72 mmol) and disuccinimide (175 mg, 1.08 mmol) was made using acetonitrile (MeCN) (3.4 mL) and stirred for 11 hours at 30 °C. The solvent was evaporated off under reduced pressure and the residue was dried in vacuum. The crude product mixture, a viscous yellow oil, containing the product was used without further purification. Mass spectral analysis of the mixture was done to observe the expected m/z 341 $[M+H]^+$.

Modification of the electrode with avidin or nitro-avidin; loading the modified electrodes with the iminobiotinylated MP-11 or with the biotinylated fluorescent dye (fluorescein).

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 avidin (5 mg/mL in HEPES buffer pH 8.5) and left for 1 h in the dark with 100 % humidity. Further, the electrodes were rinsed with 3 mM HEPES pH 8.5 three times for 2-3 minutes on an orbital shaker at medium mixing. The avidin-modified electrodes were cast with 10 µL of iminobiotin succinimide ester (0.5 mM in 3 mM of HEPES pH 8.5) and left for 1 hr in the dark with 100% humidity. Further, the electrodes were rinsed with 3 mM HEPES pH 8.5 three times for 2-3 minutes on an orbital shaker at medium mixing. The PBSE/avidin/iminobiotin succinimide ester-functionalized electrodes were cast with 10 µL of microperoxidase (MP-11) (1 mg/mL in 3 mM of HEPES buffer, pH 8.5) and left for 0.5, 1 and 2 hrs in the dark with 100% humidity. The electrodes were rinsed with 3 mM HEPES buffer, pH 8.5, five times for 2-3 minutes on an orbital shaker at medium mixing. The MP-11 leakage from the modified electrode was monitored of each washing solution by testing for the peroxidase activity through the addition of 40 µL ABTS (0.5 mM in 100 mM HEPES pH 7.0) and 20 µL H_2O_2 (0.1 M in 100 mM HEPES buffer, pH 7.0), any activity showed a color change to a green/blue coloration. Washing was continued until no color change was observed. Then, after washing out the weakly bound MP-11, only the immobilized MP-11 bound through iminobiotin-avidin linker was left at the electrode surface. Once washing was complete, the electrode was used in the further experiments with the iminobiotin-MP-11 functionalized electrode.
The buckypaper electrodes were modified with nitro-avidin in a similar procedure, using nitro-avidin instead of the "normal" avidin and 3 mM HEPES buffer, pH 7.0. Then, the modified electrodes were loaded with the biotinylated fluorescent dye (fluorescein).

Optimization of the immobilization time of MP-11 onto buckypaper/PBSE/avidin/iminobiotin succinimide ester.



(**A**) (**B**)

Figure ESM9. Optimization of time of MP-11 immobilization onto buckypaper/PBSE/avidin/iminobiotin succinimide ester. (**A**) Direct progression of the biocatalytically oxidized ABTS (green color) onto biofunctionalized electrodes with different time

of MP-11 immobilization (30; 60 and 120 min). (**B**) The same cuvettes after removing the electrodes and mixing the reagent solution.

As can be seen above (Figure EST9), the electrodes prepared were tested to indicate whether the applied MP-11 biocatalyst remained active whilst immobilized on the surface. The times indicated illustrate the amount of time each electrode was incubated following the immobilization of MP-11 as stated in the casting steps. No other difference was made between the electrodes and due to MP-11 being added following iminobiotin, the effect of immobilization and the time it takes can solely be determined from surface activity. The surface activity shows that the electrodes that were incubated for at least 1 hour following the addition of the MP-11 showed the best activity and the following picture, again illustrating the time of incubation, shows the colour change once the solution is mixed. The color change indicates a biocatalytic conversion of ABTS to its oxidized form with the simultaneous consumption of H_2O_2.

Electrochemically triggered release of MP-11.

For release, the time profile for taking samples was set up so that 2 samples were taken before application of the potential at 30 and 60 mins, and then every 20 mins after the application of the potential until a total of 160 mins have elapsed. The sample taken from each step was 200 µL, whereupon the exact same volume of the appropriate buffer solution was put back to keep the total volume the same. The samples were refrigerated and stored until analysis was done using a UV spectrometer that measured the UV absorbance change over 10 mins. Each sample (160 µL) was placed in the analysis tray for automatic sample measurement at approximate 0.5 min time intervals and the data of both was used for enzymatic activity determination. Prior to starting the analysis, 40 µL ABTS (0.5 mM in 100 mM HEPES buffer, pH 7.0) and 20 µL H_2O_2 (0.1 M in 100 mM HEPES buffer pH 7.0) were simultaneously added to each of the wells using multichannel pipette, the contents immediately mixed and the analysis initiated. UV measurement gave absorbance measured for each sample at fixed intervals over the total of the analysis time. The enzymatic activity was determined by manually calculating the curve absorbance change per min in the linear frames of its OD exponential growth. The MP-11 activity of each collected fraction (corresponding to different experiment time) was calculated as ΔOD/min at λ = 420 nm and is plotted against the time.

Analysis of H_2O_2 potentially produced upon electrochemical reduction of O_2 and assay of the MP-11 activity.

The O_2 electrochemical reduction results in production of H_2O_2 (intermediate product) upon $2e^-$-transfer process or production of H_2O upon $4e^-$-transfer process. Both products can be generated simultaneously with different ratios or one of them can be a dominated product depending on electrochemical conditions (applied potential, electrode material, etc.). In order to check if H_2O_2 is produced in meaningful concentrations, we collected samples from the bulk solution after performing electrochemical O_2 reduction and performed the enzyme assay with HRP/ABTS according to the standard conditions, Figure ESM10A. Notably, the H_2O_2 production was not observed with the used assay test. This means that under the used experimental conditions H_2O

is a dominated product of the O_2 reduction, while H_2O_2 is not produced in the amount detectable by the used assay method.

A similar assay was performed for the analysis of MP-11 biocatalytic activity. In this case H_2O_2 was added to the assay solution, Figure ESM10B. The exact conditions of the assay procedure are detailed in the preceding section. Structures of the ABTS (both initial reduced and catalytically oxidized forms) are shown in Figure ESM10C. Structure of MP-11 (without the biotinylated unit) is shown in Figure ESM10D. Figure ESM10E shows the absorbance change observed upon catalytic oxidation of ABTS in the assay procedures.

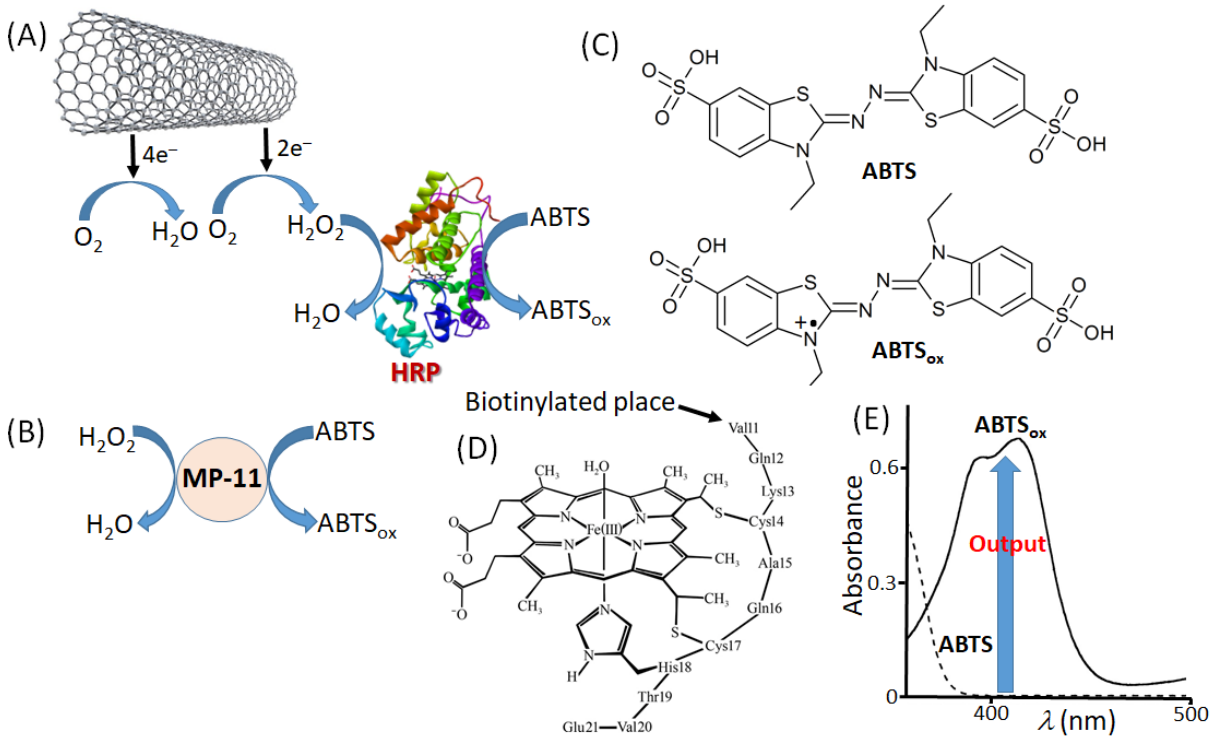


Figure ESM10. Schematically shown analysis of H_2O_2 potentially produced by the electrochemical reduction of O_2 (A) and produced upon reduction of O_2 catalyzed by MP-11 (B). (C) The structures of the initial (reduced) state of ABTS and its oxidized form ($ABTS_{ox}$). (D) The structure of MP-11 (the biotinylated end of the oligopeptide chain is shown). (E) The absorbance spectrum change upon oxidation of ABTS in the reaction catalyzed by horseradish peroxidase enzyme (HRP) or by MP-11.

Visualization of local (Interfacial) pH change

To analyze formation of a local pH-gradient layer at the buckypaper electrode surface upon applying corresponding potentials (-0.4 V for O_2 reduction or 0.2 V for ascorbate oxidation) we used confocal fluorescent microscopy combined with two different pH sensitive fluorescent dyes,

namely rhodamine-6-aniline (3',6'-*bis*(diethylamino)-2-phenylspiro[isoindoline-1,9'-xanthen]-3-one (R6H)[6] and 3,4'-dihydroxy-3',5'-*bis*-(dimethylaminomethyl)flavone (FAM345).[7] R6H was used for the acidic pH range (from 7 to 3), while FAM345 was used for the alkaline pH range (from 7 to 11). R6H was detected at 580–630 nm upon excitation at 561 nm and FAM345 was detected at 470–600 nm excitation at 405 nm. The visualization of acidic layer was performed in solution of 13 µM R6H dye with 1 mM sodium ascorbate, 100 mM Na_2SO_4 in 3 mM HEPES, pH 7.0; the alkaline layer was visualized with the same FAM345 concentration (13 µM) and the same solution, but without ascorbate addition. The images of the thin layers with electrochemically induced pH changes are shown in Figure ESM11. The images allowed semi-quantitative determination of the acidic pH (ca. pH 4) and of the alkaline pH (ca. pH 10) using the calibration scale reported recently.[8] It is interesting to note that the thickness of the layers with the pH changes was ca. 500 µm, that is clearly visible in the fluorescent images obtained with a confocal fluorescent microscope.

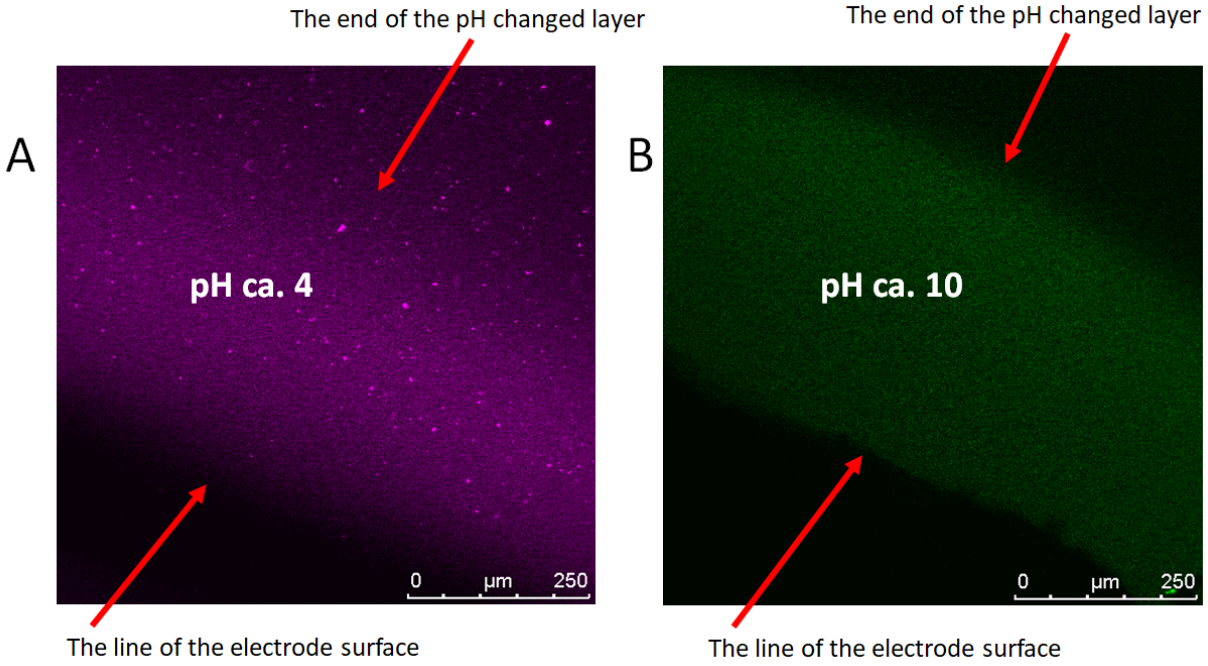


Figure ESM11. Confocal microphotography visualizing a local pH-gradient layer formed at the buckypaper electrode surface at applying potentials. (A) Acidic pH layer obtained with R6H dye and 1 mM sodium ascorbate at applied 0.2 V (160 s). (B) FAM345 dye upon generated alkaline pH layer at applied -0.4 V (160 s).

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