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Electronic Supplementary Information

Electrochemical DNA sandwich assay with a lipase label for attomole detection of DNA

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Materials. The DNA capture probe, target DNA and reporter probe sequences were synthesized by DNA Technology A/S, Risskov, Denmark. All solvents and chemicals except 5´-biotin phosphoramidite were purchased from Sigma-Aldrich, Germany, and used without further purification. 5´-Biotin phosphoramidite (1-N-(4,4´-dimethoxytrityl)-biotinyl-6-aminohexyl]-2-cyanoethyl-(N,N-diisopropyl)-phosporamidite, catalog number 10-5950-xx) was from Glen Research, USA. Quantum dot nanocrystals, Qdot®Streptavidin conjugate, with a fluorescence emission maximum at 655 nm (Q10121MP), and streptavidin-coated magnetic beads Dynabeads® MyOne™ Streptavidin T1 (2.8 and 1 μm diameter, supplied as 10 mg ml^{-1} suspension in PBS, pH 7.4/0.1% BSA/0.02% NaN_3, binding affinity of 200 and 400 pmol of biotin-conjugated ssDNA per 1 mg of beads, correspondingly) were purchased from Invitrogen, USA.
Lipase B *Candida antarctica*, recombinant form from *Aspergillus oryzae* (lyophilized, 9 U mg^{-1}) was from BioChemica and a solution of recombinant lipase from *Thermomyces lanuginosus* (100 U mg^{-1}) was from Novozymes; both were supplied by Sigma-Aldrich. BiotinTag™ Micro Biotinylation Kit (catalog number B-TAG) and streptavidin from *Streptomyces avidini* were also from Sigma-Aldrich.
18.2 MΩ Millipore water (MilliQ) was used throughout the work.

Synthesis of 9-mercaptononyl 4-ferroceneamidobutanoate. The compound was synthesized according to the following reaction scheme (Figure 1S). The details of experimental procedure and synthetic steps are specified below.

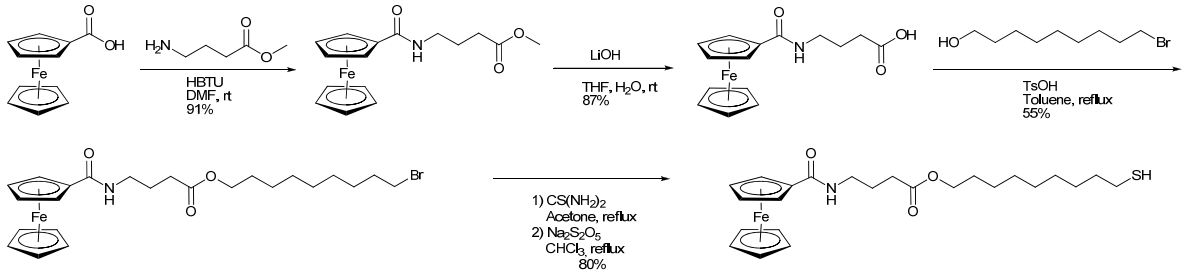

Figure S1. Reaction scheme for synthesis of 9-mercaptononyl 4-ferroceneamidobutanoate. The molecule was immobilized on the Au electrode through the terminal thiol group.

General procedures. For air and water sensitive reactions standard Schlenk techniques were employed using argon as the inert atmosphere. Flash chromatography was performed using Merck® silica gel 60 (230-400 mesh). THF was distilled from sodium and benzophenone, and Et_3N from CaH_2. All other solvents were HPLC grade quality. NMR spectra were recorded at 400 mHz (^{1}H NMR) or 100 MHz (^{13}C

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NMR) on Varian® Gemini systems and calibrated to the residual solvent peak. Coupling constants (*J*) are given in Hz. MS spectrums were obtained on a Micromass LC-TOF spectrometer. Melting points are uncorrected. Reverse-phase High Performance Liquid Chromatography (RP-HPLC) was performed on a Hewlett Packard Agilent 1100 Series fitted with a phenomenex® C18Clarity 3u Oligo-RP 50 x 4.6 mm column. The following conditions were applied: T=25°C, flow= 1.0 mL/ min Aqueous triethylammonium acetate, 0.1 M, pH 7.0 /MeCN

Methyl 4-ferroceneamidobutanoate. HTBU (660 mg, 1.74 mmol) and 4-aminobutyrate hydrochloride (267 mg, 1.74 mmol) were added to a solution of ferrocene carboxylic acid (400 mg, 1.74 mmol) and Et_3N (2.6 ml) in DMF (25 ml). The mixture was stirred for 1h at room temperature and the solvent evaporated under reduced pressure. The residue was resuspended in CH_2Cl_2 and washed with 5% $NaHCO_3$, then with 1M HCl and finally with water. The organic phase was dried over $MgSO_4$ and evaporated under vacuum to yield an orange solid (520 mg, 91%).
^{1}H NMR ($CDCl_3$): δ (ppm) 6.25 (bs, 1H), 4.67 (t, *J* = 1.8 Hz, 2H), 4.32 (t, *J* = 1.8 Hz, 2H), 4.18 (s, 5H), 3.68 (s, 3H), 3.41 (q, *J* = 6.8 Hz, 2H), 2.44 (t, *J* = 6.8 Hz, 2H), 1.92 (qn, *J* = 6.8 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ (ppm) 174.4, 170.2, 76.2, 70.5, 69.8, 68.1, 52.0, 39.3, 31.8, 24.8. MS calcd. 352.0612 ($[M + Na]^+$), found 352.0609 mp 122.8-123.6°C.

4-Ferroceneamidobutanoic acid. LiOH (945 mg, 39.5 mmol) was added to a solution of methyl 4-ferroceneamidobutanoate (507 mg, 1.54 mmol) in THF (20 ml) and water (5 ml) and the suspension was stirred over night at room temperature. The THF was evaporated under reduced pressure and the remaining aqueous solution adjusted to pH 5 with AcOH and the mixture was then extracted with EtOAc. The combined organic phases were washed with brine, dried over Na_2SO_4 and the solvent evaporated under reduced pressure. The residue was filtered through a 5 cm silica plug (MeOH/EtOAc 1:19 → MeOH/EtOAc 1:1) to yield the product as red crystals (415 mg, 86%).
^{1}H NMR (MeOD): δ (ppm) 4.79 (t, *J* = 1.8 Hz, 2H), 4.36 (t, *J* = 1.8 Hz, 2H), 4.18 (s, 5H), 3.34 (t, *J* = 7.2 Hz, 2H), 2.37 (t, *J* = 7.2 Hz, 2H), 1.85 (qn, *J* = 7.2 Hz, 2H). ^{13}C NMR (MeOD): δ (ppm) 177.0, 173.4, 76.6, 71.7, 70.7, 69.3, 39.9, 32.3, 26.0. MS calcd 338.0456 ($[M + Na]^+$), found 338.0453 mp 146.5-147.8°C.

9-Bromononyl 4-ferroceneamidobutanoate. The compound was synthesized using a modified literature procedure (1). A solution of 4-ferroceneamidobutanoic acid (393 mg, 1.25 mmol), TsOH (238 mg, 1.25 mmol) and 9-bromononanol (293 mg, 1.25 mmol) in dry toluene (25 ml) was refluxed for 4.5 h under argon using a Dean-Stark head. The solvent was evaporated under reduced pressure and the residue purified by flash chromatography (hexane/EtOAc 2:1) to yield the product as orange crystals (357 mg, 55%).
^{1}H NMR (MeOD): δ (ppm) 7.97 (bs, 1H) 4.79 (t, *J* = 1.8 Hz, 2H), 4.40 (t, *J* = 1.8 Hz, 2H), 4.20 (s, 5H), 4.08 (t, *J* = 6.8 Hz, 2H), 3.42 (t, *J* = 6.8 Hz, 2H), 3.34 (q, *J* = 6.8 Hz, 2H), 2.43 (t, *J* = 6.8 Hz, 2H), 1.89 (qn, *J* = 6.8 Hz, 2H), 1.82 (qn, *J* = 6.8 Hz, 2H), 1.63 (qn, *J* = 6.8 Hz, 2H), 1.42 (qn, *J* = 6.8 Hz, 2H), 1.31 (m, 8H). ^{13}C NMR (MeOD): δ (ppm) 174.0, 170.4, 76.2, 70.4, 69.8, 68.1, 64.9, 39.3, 34.2, 32.8, 32.1, 29.3, 29.2, 28.7, 28.6, 28.2, 25.9, 24.8. MS calcd. 542.0969 ($[M + Na]^+$), found 542.0959 mp 65.9-66.6°C.

9-Mercaptononyl 4-ferroceneamidobutanoate. The compound was synthesized using a modified literature procedure (1). A solution containing 9-bromononyl 4-ferroceneamidobutanoate (280 mg, 0.54 mmol) and thiocarbamide (164 mg, 2.15 mmol) in dry acetone (15ml) was refluxed under argon for 40h. The solvent was evaporated and upon addition of $Na_2S_2O_5$ (190 mg, 1.09 mmol), $CHCl_3$ (20 ml) and water (10 ml) the mixture was vigorously stirred and refluxed for 3 h. The organic phase was washed twice with water, dried over $MgSO_4$, evaporated under reduced pressure and purified by flash chromatography (hexane/EtOAc 2:3) to yield both the thiol (159 mg, 62%) and the disulfide (45 mg, 18%) as orange powers.

^{1}H NMR ($CDCl_3$): δ (ppm) 6.26 (bs, 1H) 4.66 (t, *J* = 1.8 Hz, 2H), 4.30 (t, *J* = 1.8 Hz, 2H), 4.16 (s, 5H), 4.04 (t, *J* = 6.8 Hz, 2H), 3.39 (q, *J* = 6.8 Hz, 2H), 2.47 (q, *J* = 6.8 Hz, 2H), 2.40 (t, *J* = 6.8 Hz, 2H), 1.90 (qn, *J* = 6.8 Hz, 4H), 1.56 (qn, *J* = 6.8 Hz, 2H), 1.30 (qn, *J* = 6.8 Hz, 2H), 1.24 (m, 8H). ^{13}C NMR ($CDCl_3$): δ (ppm) 174.0, 170.4, 76.2, 70.4, 69.7, 68.1, 64.9, 39.2, 34.0, 32.0, 29.4, 29.2, 29.0, 28.6, 28.3, 25.9, 24.8, 24.7 496.1585. MS calculated 496.1585 ($[M + Na]^+$), found 496.1585 mp 68.3-69.5°C.

Catalytic cleavage of 9-mercaptononyl 4-ferroceneamidobutanoate by lipase. Lipase from either *Thermomyces lanuginosus* or *Candida Antarctica* (9μL of a stock solution, 4.3 U/ml and 2.4 U/ml, respectively) was diluted by PBS, pH 7 (9 μL), and mixed with a solution of 0.2 mg of 9-mercaptononyl 4-ferroceneamidobutanoate in MeOH (2 μL). The mixture was incubated with gentle shaking for 16 h at room temperature. MeCN (40 μL) was added and the solution was HPLC analyzed. HPLC data: (0-80%, 30 min) rt. 27.24 (9-mercaptononyl 4-ferroceneamidobutanoate), 10.54 and 10.13 (fragments). As can be seen from Figure 2S, the synthesized ferrocene-terminated alkanethiol ester is catalytically cleaved by lipase.

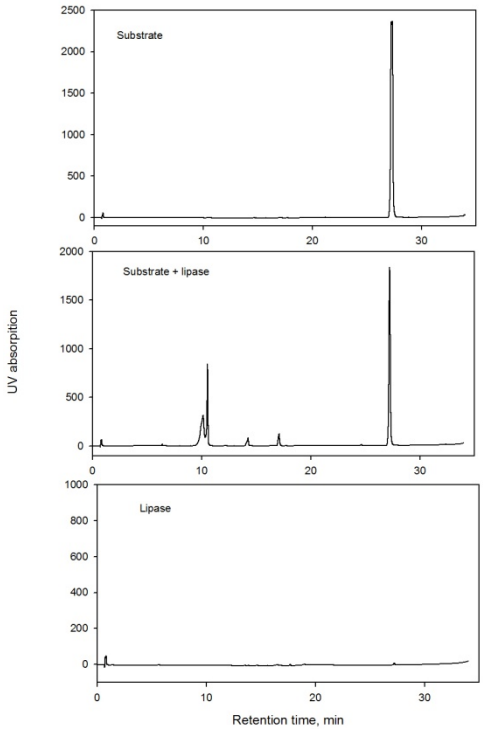

Figure S2. HPLC data for the ferrocene (Fc)-terminated alkanethiol ester (substrate) cleavage by lipase.

Electrode modification with alkanethiols. Prior to modification, the gold disk electrodes (CH Instruments, USA, diameter 0.2 cm) were mechanically polished to a mirror luster stepwise in 1 μm diamond- and in 0.1 μm alumna slurry (both from Struers, Denmark) on microcloth (Buehler, Germany), ultrasonicated in water bath for 5 min, electrochemically polished by cycling in 1 M H_2SO_4 and further kept in absolute ethanol for 30 min (*1*). The electrode surface area was determined after electrochemical polishing procedure, from the peaks of surface oxides reduction in 1 M H_2SO_4.
200 nm-thick 500 nm×500 nm gold electrodes of the 8-microelectrode array used in another set of experiments (produced using standard microfabrication techniques on 3-inch <100> n-type silicon wafers (*2*) at the Scottish Microeletronics Center, Edinburgh, UK, Figure 3S) were cleaned in acetone, dried in the flow of Ar, then cleaned for 2 min in a freshly prepared Pyrnaha solution for 2 min [**NB**: Piranha solution is highly hazardous one and reacts violently with organic materials; precautions should be taken at all the times when handled] and further kept in absolute ethanol for 30 min under gentle shaking. Modification of the gold electrodes with 9-mercaptonon-1'-yl 4-ferroceneamidobutanoate (Fc alkanethiol ester) and other alkanethiol SAMs was performed by incubation of the electrodes for 2 h in their 10 mM solutions in ethanol. Dilution of Fc-terminated SAM on gold electrodes was also performed with 1-

hexanethiol, 1-propanethiol, 1-butanethiol, and 2-ethylhexanethiol, following the adsorption of alkanethiols from the mixture with Fc-alkanethiol ester. After modifications, the SAM-modified electrodes were thoroughly rinsed in ethanol and used in further experiments or kept in ethanol, in the dark, at 4^{o}C.

Instrumentation and procedure. Cyclic voltammetry (CV) experiments were done with a three-electrode potentiostat AUTOLAB PGSTAT 30 (Eco Chemie B.V., Utrecht, the Netherlands) equipped with GPES and FRA 4.9.006 software. An Ag/ AgCl(3 M KCl) electrode was the reference and a stainless steel ring was the auxiliary electrode. The electrolyte was either 1 M H_2SO_4 or aqueous 20 mM phosphate buffer solution, pH 7, containing 0.15 M NaCl (PBS).

Preparation of the lipase-biotin conjugate. Conjugation of lipases with biotin was performed with BTAG BiotinTag^{TM} Micro Biotinylation Kit (Sigma), by 30 min reaction of 1 mg of lipase in a sample with biotin modified with aminocaproate (aminocaproate provides a six-carbon spacer that reduces steric hindrance on the biotin and improves accessibility to the binding site on avidin) and activated via an ester linkage with sulfo-N-hydroxysuccinimide (BAC-Sulfo-NHS). Separation of the conjugate from the reactants was performed by gel filtration. Only lipase from *Candida antarctica* displayed catalytic activity after conjugation to biotin, and thus was used for hybridized DNA labeling.

DNA assembly on magnetic beads (MB). A DNA hybridisation assembly was performed using streptavidin-coated MB, a biotinylated capture DNA sequence , a target DNA sequence and a biotinylated reporter probe. First, 100 µl of MB were carefully washed using the Vortex system and decanted with a magnet in 3×100 µl of 20 mM phosphate buffer solution, pH 7, containing 0.15 M NaCl (PBS) and 0.1% Tween 20, and finally re-dissolved in 100µl of PBS. Then, a two-fold excess of the capture DNA was added to the solution of MB beads (0.4 nmoles of MB-streptavidin binding sites: 0.84 nmoles of DNA) and let react for 40 min, after that capture DNA-modified MB were carefully washed from non-reacted DNA (in 3×100 µl PBS) and re-suspended in 100µl PBS, containing 10 mM $MgCl_2$. Then, in one set of experiments, MB with 0.1 nmol DNA-binding capacity were mixed with from 0.1 to 100 pmol of target DNA, let react for 40 min, washed/decanted and re-suspended in equivalent amount of PBS, let to react with 0.6 nmoles of the reporter DNA, and after careful washing/decanting and re-suspending procedures, labelled with lipase by addition of 10-fold mole excess of streptavidin (40 min reaction time, then washing/decantation/re-suspension) and further addition of 4-fold excess of lipase-biotin conjugate to DNA-assembled MB, to react with the biotinylated end of the reporter probe of the hybridised sandwich through the streptavidin linker (Vortex stirring and 1 h reaction at 4^{o}C). In another set of experiments, the capture DNA-modified MB with 1 pmole DNA-binding capacity were allowed to react with from 0.01 fmole to 1 pmole of the target DNA. Throughout the experiments, the excess material was washed away while retaining the magnetic beads by the use of a magnet. PBS was used for DNA and protein assembly on MB and washing, and PBS, containing 5 mM $MgCl_2$, was used in hybridization experiments.
In DNA detection experiments, 5 µl of the 0.05-1 mg ml^{-1} lipase-labeled DNA-hybridized MB suspension (test solutions) in PBS were placed on the top of the Fc-alkanethiol ester-modified electrode and allowed to react for various times (from 10 to 60 min). In final experiments 60 min reaction time was chosen as most suitable for experiments both in low and medium DNA concentration range. With microelectronic chips, a 10 µl of test solution were placed on the top of each array electrode, having the underlying magnets. All MB/DNA concentrations were recalculated for dilutions. After careful washing with PBS, the electrodes were transferred to the electrochemical cell. The solution was equilibrated for 5 min before the CV measurements.

Fluorescence Microscopy visualization of the catalytic cleavage of the ester bond of the Fc–alkane ester SAM by lipase-DNA-hybrid-modified MB. 0.1 M solution of a 5´-biotin phosphoramidite linker in acetonitrile, specifically reacting with free hydroxyl groups, was put onto the SAM-modified microarray electrode top for 5 min and then washed with acetonitrile. Then 2% solution of TFA (triflouroacetic acid, cleaves the protection group on the biotin phosphoramidite) in acetonitrile was

allowed to react with the electrodes for 10 min and then washed away. After that 0.5 µl of QD-streptavidin conjugate and 0.5 µl PBS were put on the electrode surface, let to react for 35 min (in water-saturated atmosphere), and carefully washed away with water/PBS, pH 7. The fluorescence microscopy images were made using an Olympus BX51 with a 100 w Hg lamp and WG filter setting (570 nm Dichroic mirror, Excitation filter 510-550 nm and Barrier filter 590 nm). Images were taken at 20X magnification with identical exposure times (5000 ms) in each case.

Electrochemical characterization of the Fc-alkanethiol ester SAMs.

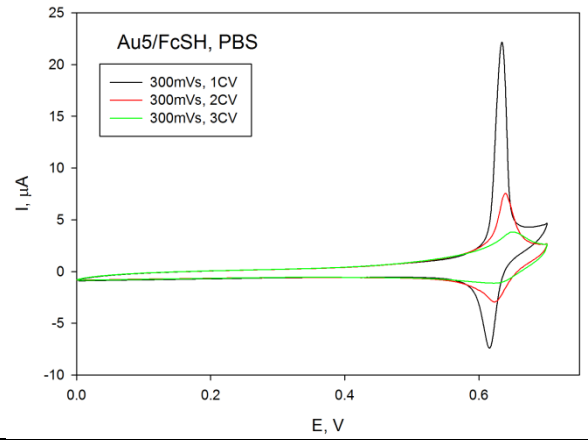

Figure S3. Cyclic voltammograms of the gold disk electrode modified by Fc-alkanethiol ester in PBS, pH 7, (1) first, (2) second and (3) third scans, scan rate 0.3V s^{-1}.

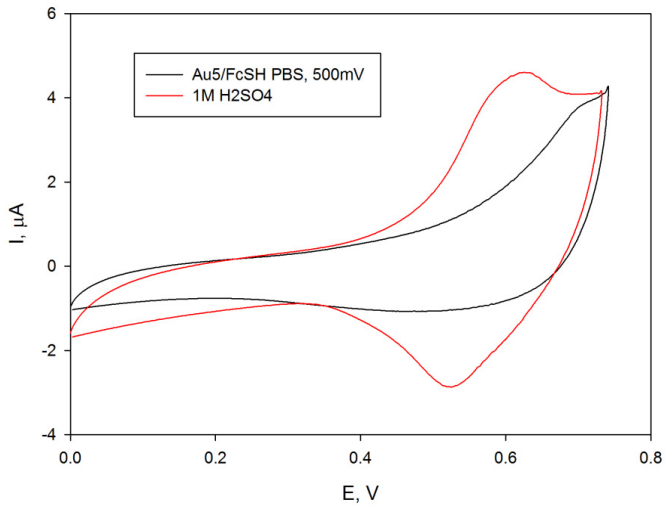

Figure S4. Cyclic voltammograms of the gold disk electrode modified by Fc-alkanethiol ester in (1) PBS, pH 7, and (2) after transfer to 1 M H_2SO_4, scan rate 0.5 V s^{-1}.

References:

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