Supporting Information

Catalysed amplification of faradaic shotgun tagging in ultrasensitive electrochemical immunoassays

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Table of contents

Contents

Materials and methods

Materials and equipment [2,5-Dioxo-1-[6-[5-(2-oxo-1,3,3a,4,6,6a-hexahydrothieno[3,4-d]imidazol-4-yl) pentanoylamino] hexanoyloxy] pyrrolidine-3-sulfonic acid sodium salt] (Biotin-LC-NHS) and biotinylating reagent (Cat # ab145611) were purchased from abcam© and used as provided. Mouse anti-Human α-Synuclein (α-Syn) capture antibody and recombinant human α-Synuclein standard antigen were purchased from R&D systems© (Cat # DY1338). Streptavidin Poly-HRP80 conjugate was obtained from Fitzgerald©. Tyramine, fibrinogen, bovine serum albumin (BSA), human serum albumin (HSA), and human serum were purchased from Sigma-Aldrich©. N-Succinimidyl Ferrocenecarboxylate (Ferrocene-NHS) was bought from TCI©. All electrochemical measurements were carried out with a 3-electrode setup using a PalmSens 4 Multi-potentiostat powered by MultiTrace©. The SPR measurements were performed on Reichert DC7200 and data collected and analysed using integrated SPRAutolink software. Screen-printed 32 carbon electrode arrays were provided by Osler© diagnostics and fitted to ProPlate® bottomless microtiter 96 well-plate. Electrode arrays are designed such that each well houses a 3 mm screen printed carbon electrode, an Ag/AgCl reference electrode and a carbon counter electrode (Figure S1). Water used throughout was ultra-purified with a resistivity of 18.2 MΩ.cm (Milli-Q® Direct/Merck Millipore®).

SPR Study of solution phase shotgun biotinylation: SPR gold chips were cleaned by immersion in fresh Piranha solution (sulfuric acid and hydrogen peroxide 3:1 mixture) for 10 min, washed with water and ethanol and dried under nitrogen. The chip was then fitted into the sample holder of the Reichert SPR and allowed to equilibrate under running PBS buffer for 15 min. Anti α-Syn antibodies (20 μg/mL in PBS) were then injected and incubated for 1 hr for passive immobilization onto the chip surface. The chip was then washed and blocked by incubation with 1% BSA in PBS for 30 min. A series of concentrations of target protein, α-Syn, aliquoted in 1% BSA in PBS were incubated with 3.0 μM Biotin-LC-NHS for 30 min. The labelled (biotin-tagged) protein samples were injected onto the antibody modified SPR chip and incubated for 5 min and then washed. Subsequently, a 100 ng/mL Streptavidin Poly-HRP80 Conjugate-Poly(HRP) (St-Poly(HRP) was injected and incubated for 5 min, then washed. Similar procedures were repeated on Anti α-Syn modified chip with non-biotinylated protein samples.

Synthesis of Ferrocene tyramide (Fc-Ty):
The synthesis of Fc-Ty was achieved by standard amide bond formation between ferrocene carboxylic acid NHS ester and tyramine as outlined in Scheme 1. As detailed below, this was carried out both on a smaller scale in situ (without product isolation, Approach 1) as well as on a larger scale including product isolation and characterisation (Approach 2). For practical reasons, we generally recommend the straight-forward synthesis and isolation of the purified Fc-Ty as a solid (Approach 2). However, it should be noted that the assay performance is identical in all cases.
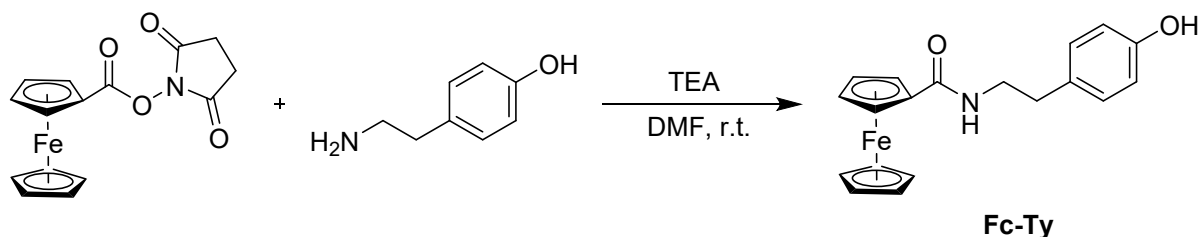
Figure S 1: Reaction pathway for the synthesis of tyramine -ferrocene by mixing Ferrocene succinimidyl ester with tyramine in DMF in basic environment.

Approach 1: In Situ Synthesis of Ferrocene tyramide (Fc-Ty):

Ferrocene carboxylic acid NHS ester (65 mg, 0.199 mmol, 1.1 equiv.) and tyramine (25 mg, 0.182 mmol, 1 equiv.) were dissolved in 5 mL anhydrous DMF. To this solution triethylamine (17 mg, 32 µL, 0.166 mmol, 0.91 equiv.) was added and reacted at room temperature for 2 h. This solution was used and stored for up to 8 weeks at 4 °C. [1]

Approach 2: Isolation and Synthesis of Ferrocene tyramide (Fc-Ty):

Ferrocene carboxylic acid NHS ester (200 mg, 0.612 mmol, 1 equiv.) and tyramine (84 mg, 0.612 mmol, 1 equiv.) were dissolved in 15 mL anhydrous DMF under N_2. To this solution triethylamine (62 mg, 85 µL, 0.612 mmol, 1 equiv.) was added and the solution was reacted under N_2 at room temperature overnight. 100 mL DCM were then added and the organic phase washed twice with water (100 mL) and once with brine (100 mL). The organic phase was then dried over $MgSO_4$ and reduced in vacuo. The product was purified by silica gel column chromatography (DCM/MeOH 97:3 (v/v)) to afford 159 mg (0.456 mmol, 75%) of Fc-Ty as an orange solid.

^{1}H NMR (400 MHz, DMSO) δ 9.15 (s, 1H), 7.79 (t, *J* = 5.7 Hz, 1H), 7.19 – 6.95 (m, 2H), 6.83 – 6.59 (m, 2H), 4.75 (t, *J* = 2.0 Hz, 2H), 4.31 (t, *J* = 1.9 Hz, 2H), 4.09 (s, 5H), 3.35 (m, overlaps with solvent), 2.70 (t, *J* = 7.4 Hz, 2H).

MS: (ESI +): 349.2 [M]+; (ESI -): 348.0 [M-H]-

^{13}C NMR (151 MHz, DMSO) δ 168.64, 155.55, 129.59, 129.42, 115.05, 76.84, 69.69, 69.23, 68.01, 40.55, 34.55.

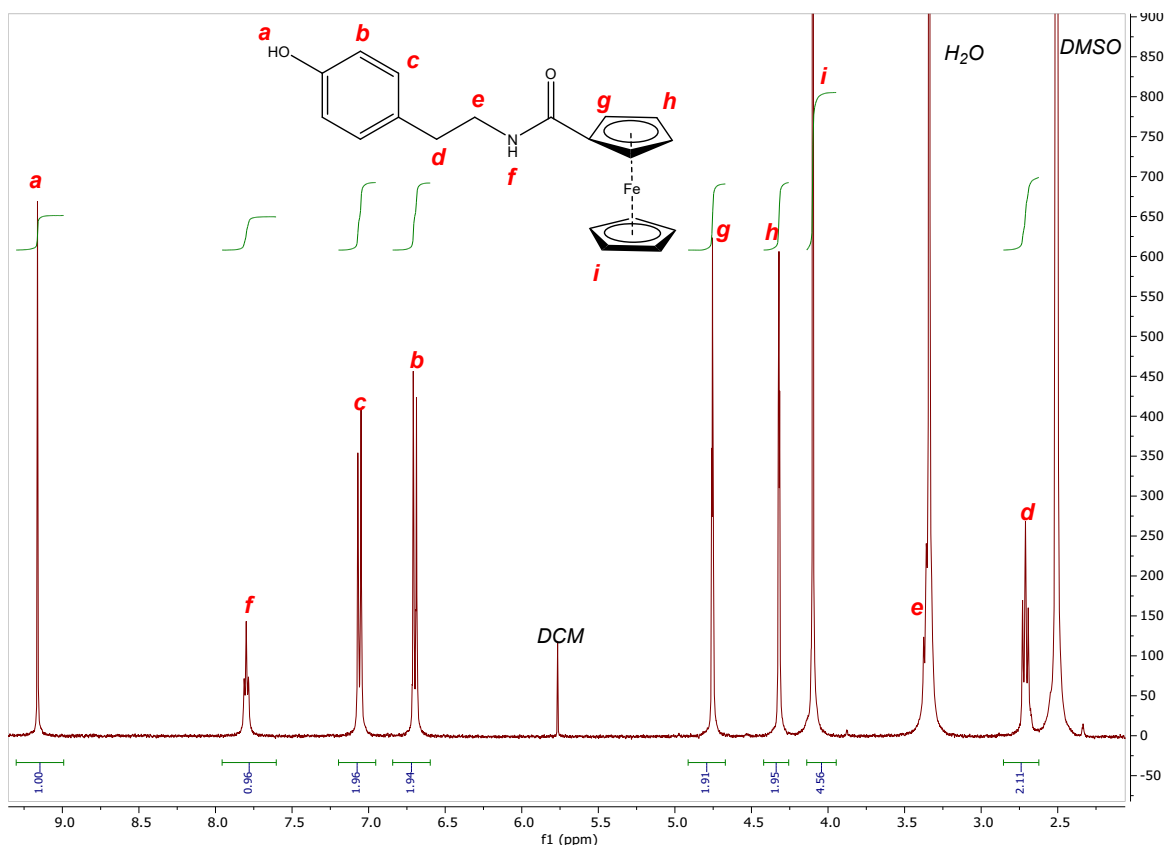**HR-MS**: (ESI+) m/z 350.0836 [M+H]$^+$; calculated for $C_{19}H_{20}FeNO_2$: 350.083

Figure S 2: ^{1}H NMR of Fc-Ty in DMSO-d_6 (400 MHz).

Preparation of Fc-Ty Tagging Solution:

The Fc-Ty reaction solution for covalent Fc-tagging of the HRP-modified interfaces was prepared by 5-fold dilution of a 40 mM DMF solution of Fc-Ty (either directly obtained by synthesis Approach 1 or prepared from solid Fc-Ty) with a 1:1 (v/v) mixture of EtOH/H$_2$O to give an overall concentration of 8 mM Fc-Ty.

Electrochemical shotgun assay: Electrode arrays were used as provided. Each array, consisting of 32 working screen-printed carbon electrodes, was washed with ethanol, dried under nitrogen and fitted into a bottomless 96 well-plate (ProPlate® microtiter plate). 50 µL of anti α-Syn antibodies (10 µg/mL) were added to each well and incubated at 4 °C overnight. Afterwards, the electrodes were washed, blocked by incubation with 100 µL of 1% BSA (in PBS) for 1 h. Protein samples (a series of concentrations of α-Syn) prepared in 1% BSA were biotinylated by incubation with 3.0 µM Biotin-LC-NHS for 30 min. When α-Syn samples were aliquoted in 1% human serum (diluted in PBS), 200 µM Biotin-LC-NHS was used to shotgun biotinylate proteins in the samples (assuming a total protein concentration of 80 mg/mL in neat human serum that is approximately 10 µM total protein for 1% human serum).[2] Arrays were then washed and 50 µL of the labelled protein samples were added to each well in quadruplicates where they were incubated for 15 min (each sample is measured on 4 different electrodes/wells). After incubation, electrodes were washed with PBS-T20 (PBS with 0.05 % Tween-20), incubated with 50 µL of 100 ng/mL St-Poly(HRP), washed and incubated with 50 µL of 8mM Fc-Ty (in 1 mM H$_2$O$_2$) for 5 min to allow the HRP-

catalysed tethering of ferrocene onto adjacent proteins. Finally, electrodes were washed with PBS-T20 and integrated with a specially designed 32-electrode connector. Square wave voltammograms (SWV) were recorded in 0.1 M $KClO_4$ between 0.0 and 0.6 V vs Ag/AgCl reference electrode. The SWV pulse amplitude was 0.1 V with step increment of 0.01 V over 0.01 s intervals at 25.0 Hz frequency. Electrochemical signals were measured using a Palmsense 4-channel multi-potentiostat. The 32 electrodes were analysed across 8 successive measurements; 4 electrodes/measurement.

Specificity studies: Electrodes in screen printed carbon arrays functionalized with anti α-Syn antibodies were exposed to varying concentrations of common interfering proteins that were shotgun biotinylated in a procedure similar to abovementioned. After incubation, electrodes were washed with PBS-T20 and incubated with 50 μL of 100 ng/mL St-Poly(HRP), then washed with PBS-T20 and incubated with 50 μL of 8 mM Fc-Ty (in 1 mM H_2O_2). The SWV signals were measured and compared to those generated after incubating electrodes to 40 pg/mL α-Syn.

Spike recovery studies: A series of different concentrations of α-Syn were spiked into 1% human serum (HS) and shotgun biotinylated. These samples were then assayed using the same procedures described for electrochemical shotgun assay. The measured electrochemical signals were then used to back-calculate the found α-Syn concentration using the calibration data from recombinant α-Syn standards.

SPR Studies

The SPR studies were performed to confirm the shotgun biotinylation of proteins and the specificity of the ST-poly(HRP) binding to captured biotinylated α-Syn compared to the native α-Syn. Figure S2 demonstrates a biotin-specific binding of the ST-poly(HRP) resulting in a significant SPR signal boost (compared to non-labelled α-Syn) due to the large molecular weight difference (ST-Poly(HRP) is approximately 1.8×10^4 KDa and α-Syn is ≈ 14.5 KDa).
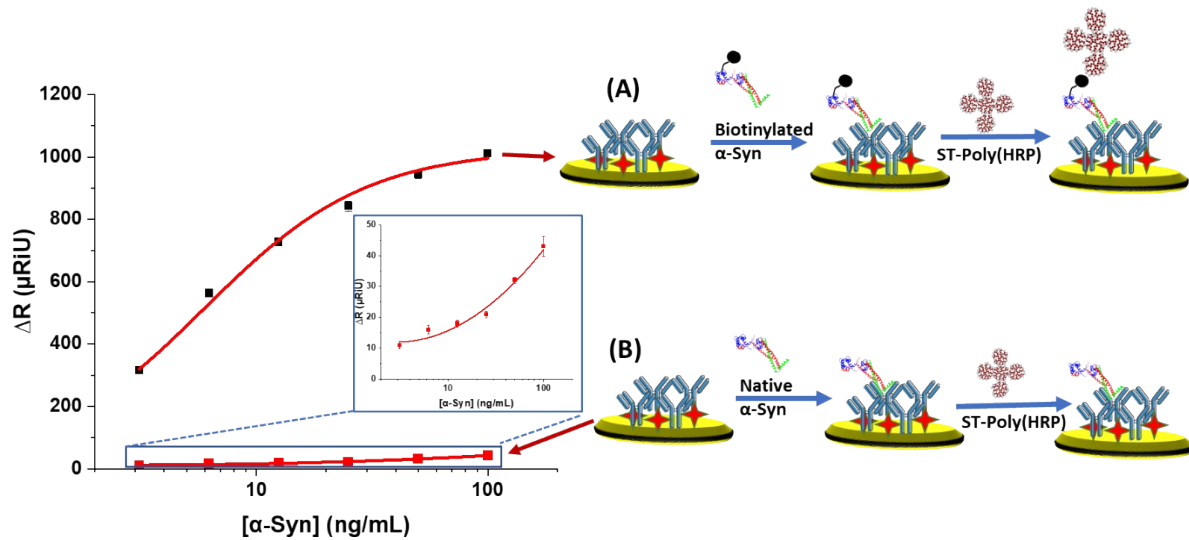

Figure S 3: SPR analysis of the successive incubation of increasing concentrations of (A) shotgun biotinylated α-Syn and (B) native (non-biotinylated) α-Syn on anti α-Syn modified SPR chips followed by injection of 100 ng/mL St-Poly(HRP). While both biotinylated and non-biotinylated proteins were recruited onto the chips, only the former shows a concentration dependent response after exposure to St-Poly(HRP) indicating both successful shotgun biotinylation and specificity in terms of HRP recruitment. The inset shows the response of anti α-Syn modified SPR chips to α-Syn samples with increasing concentrations. Error bars represent one standard deviation over 5 sampling points across the SPR sensogram.

The electrochemical signal of HRP-catalysed deposition of Ferrocene-tyramine on HRP modified electrodes

To confirm the specificity of the tyramine-mediated ferrocene deposition, electrodes were decorated with different concentrations of HRP through simple physisorption on bare standard glassy carbon disc electrodes (3 mm). The ferrocene signal was absent in bare unmodified electrodes (after incubation with Fc-Ty) while HRP-modified electrodes showed concentration-dependent voltammetric signatures.
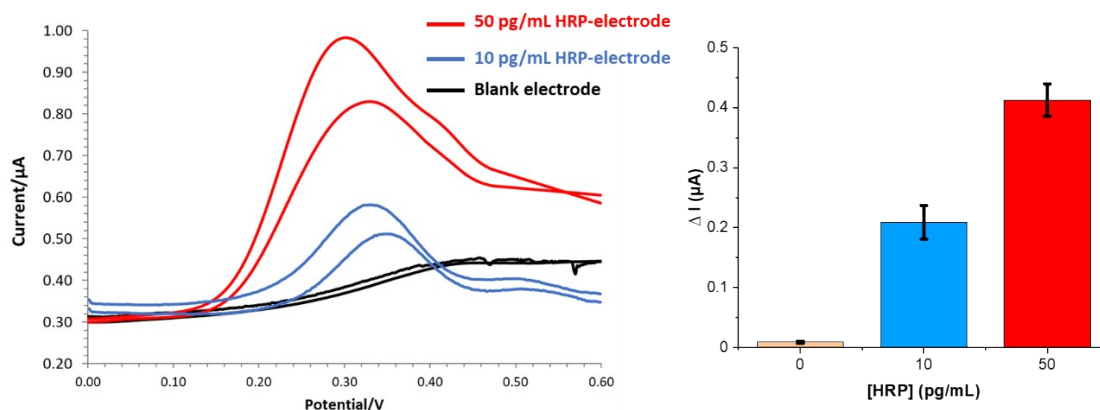

Figure S 4: Electrochemical signal after 10 min incubation of Fc-Ty on 10 pg/mL and 50 pg/mL HRP immobilized on 3 mm glassy carbon disk electrodes as compared to blank electrode. SWV were measured in 0.1 M KCLO4 after washing electrodes 3X with PBS-T20. Error bars represent one standard deviation (n=2).
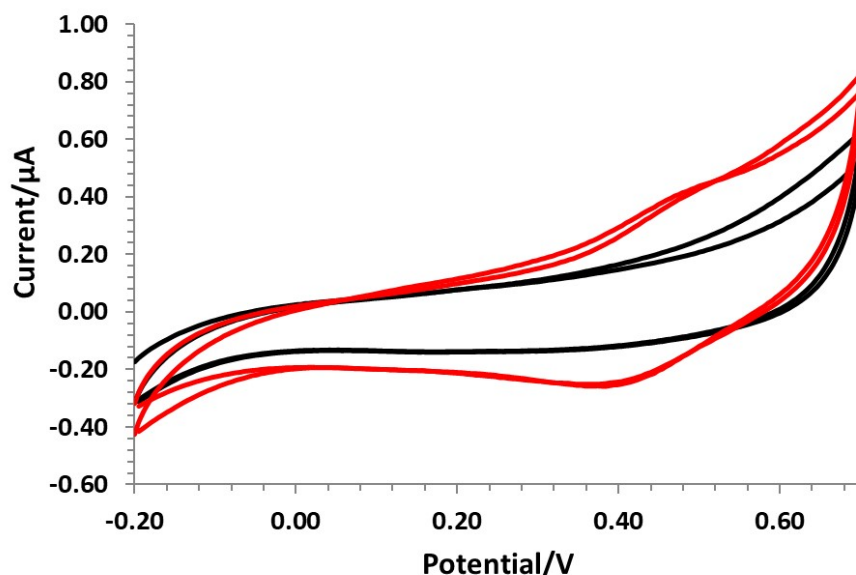

Figure S 5: Cyclic voltammograms of screen-printed electrodes coated with 10 pg/mL HRP after exposure to Fc-Ty (20 mM) alone (black) and to a solution containing 20 mM Fc-Ty and 25 mM hydrogen peroxide. The absence of redox peaks in absence of hydrogen peroxide highlights the critical role of H_2O_2 to enable the HRP- catalysed tyramine-mediated ferrocene deposition on the electrode surface.

Differntial pulse voltammetry

In order to assess the relative performance of the assay using square wave voltammetry (c.f differential pulse voltammetry (DPV), the same assay procedures described previously for SWV were used. DPV based assays showed, of course, a similar increment in the peak current with increasing α-Syn concentration but with slightly lower sensitivity (100 fg/mL LOD; a 4 fold higher than that of the SWV assay). This lower sensitivity is very likely to be reflective of the higher background currents inherent in DPV (ca. 1.3 µA compared to a much lower background with SWV of approximately 0.2 µA).
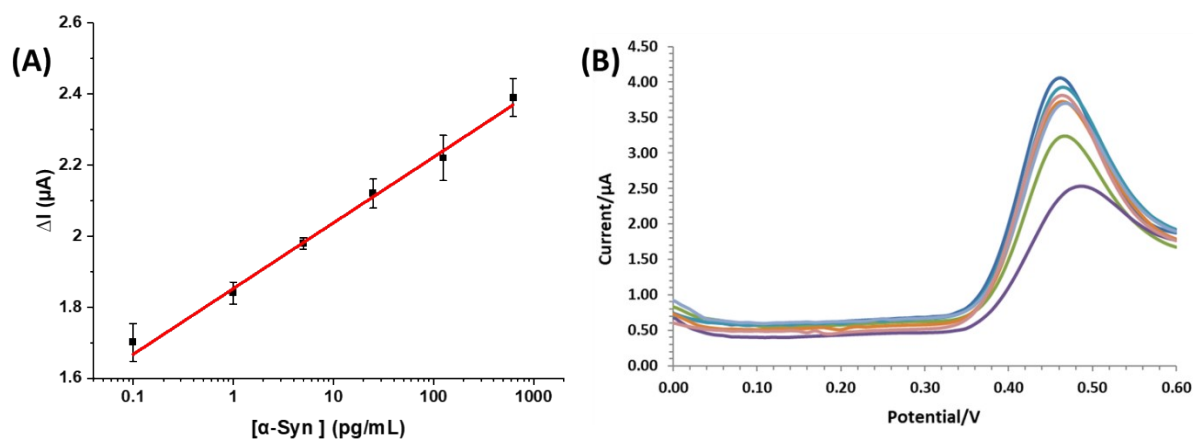

Figure S 6: Calibration data obtained after 5 min incubation of Fc-Ty followed by DPV measurement of the voltammetric fingerprint of deposited ferrocene on electrode surface. (A) calibration generating a dynamic range between 100 fg/mL to 625 pg/mL and (B) Representative raw data for the DPV measurements of the deposited ferrocene. Error bars represent standard deviation from 4 different measurements on 4 different electrodes.
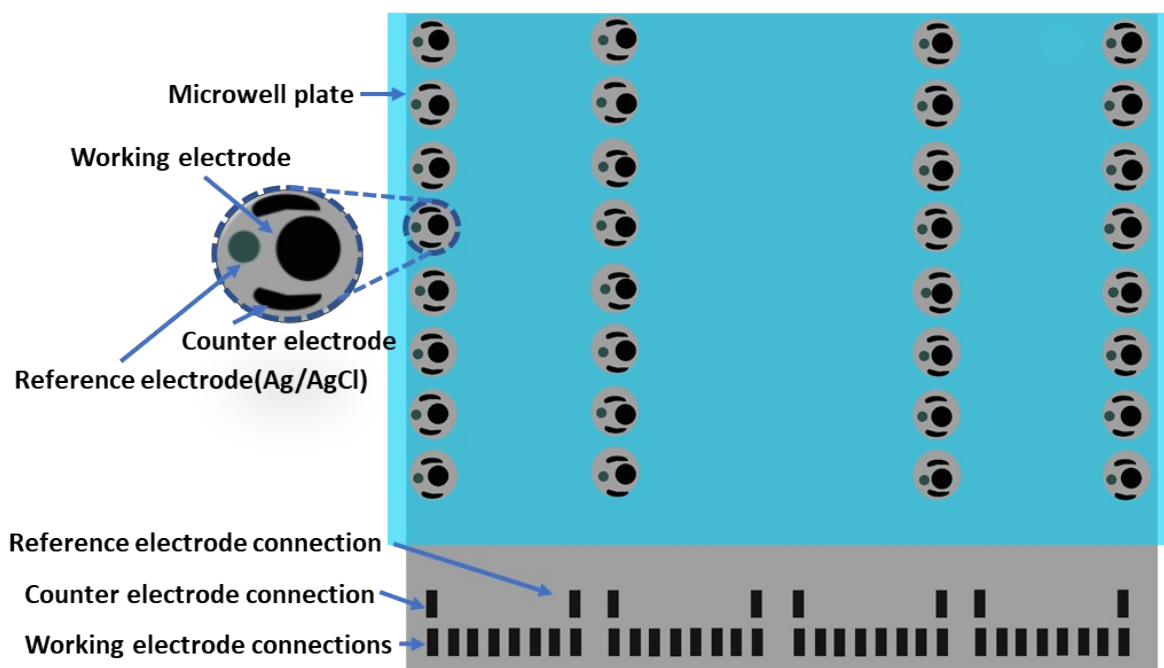
Electrode array design

Figure S 7: Schematic depiction of electrode array. The array is designed to fit into a bottomless standard 96 micro well-plate in a way that each well houses a 3 mm screen-printed carbon electrode, a screen-printed carbon counter electrode and Ag/AgCl reference electrode.

Spike recovery of α-Syn in 1% human serum

α-Syn spiked in 1% human serum (diluted in PBS) was analysed in 4 replicates for each concentration using the same assay protocol described for standards. The results showed an excellent correlation to spiked concentrations over a wide range from 128 fg/mL up to 400 pg/mL.
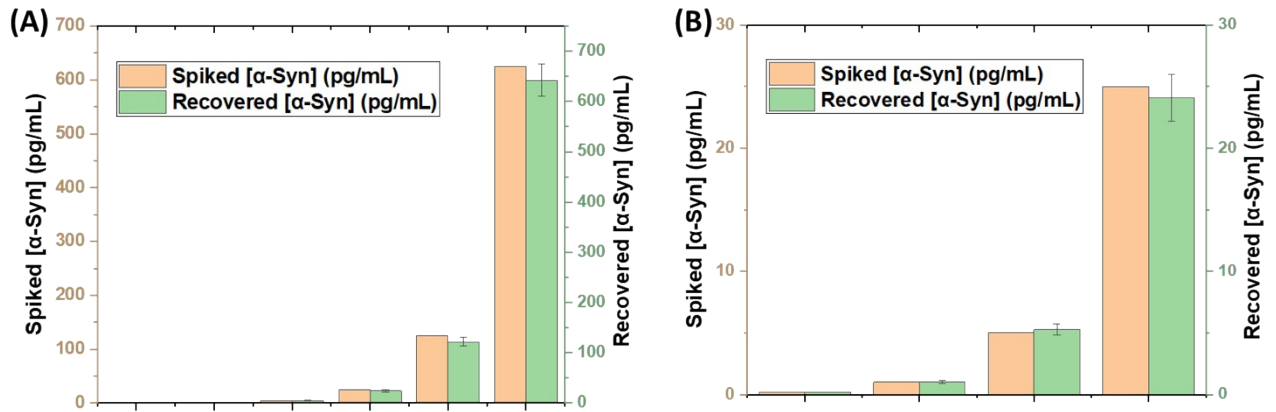

Figure S 8: Data from analysis of spiked 1% human serum containing different α-Syn concentrations. (A) bar chart comparison between spiked concentrations and found concentrations of α-Syn in a full range between 128 fg/mL and 400 pg/mL. (B) Same data presented in (A) but only covering the range between 128 fg/mL and 25 pg/mL. Error bars represent one standard deviation (n=4).

Raw α-Syn recovery data

Table S 1: Analyses of spiked samples in human serum compared to the spiked concentrations and calculated percentage recovery. Recoveries were between 97-106 % indicating excellent assay accuracy.

Spiked α-Syn Conc. [pg/mL]	Recovered α-Syn [pg/mL]	Percent Recovery
0.2	0.20 (± 0.02)	98.9
1	1.04 (± 0.12)	103.5
5	5.27 (± 0.45)	105.4
25	24.08 (± 1.89)	96.3
125	122.00 (± 8.94)	97.6
625	642.50 (± 32.5)	102.8

References

1. A. H. N. Hopman, F. C. S. Ramaekers and E. J. M. Speel, *J. Histochem. Cytochem.*, 1998, **46**, 771-777.
2. M. Leeman, J. Choi, S. Hansson, M. U. Storm and L. Nilsson, *Anal. Bioanal. Chem.*, 2018, **410**, 4867-4873.