Supporting Information

Facile impedimetric analysis of neuronal exosome markers in Parkinson's disease diagnostics

Ying Fu,[1‡] Cheng Jiang,[2‡] George K Tofaris,[2*] Jason Davis[1*]

[1] Department of Chemistry, University of Oxford, Oxford OX1 3QZ, United Kingdom

[2] Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

*jason.davis@chem.ox.ac.uk or george.tofaris@ndcn.ox.ac.uk

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Materials and methods

All chemical reagents were used as received. Potassium ferricyanide, potassium ferrocyanide, 3-mercaptopropionic acid (3-MPA), 2-mercaptoethanol (2-MU), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC), N-hydroxysuccinimde (NHS), Triton X-100 (TX), bis(carboxymethyl)trithiocarbonate (BisCTTC) were obtained from Sigma-Aldrich (Gillingham, U.K.). Commercial electrochemiluminescence (ECL) detection plates with linkers and ruthenium tags were ordered from Meso Scale Discovery (MSD, United States). Sera-Mag Carboxylate modified magnetic beads (24152105050250) were purchased from GE Healthcare and used as controls. (Buckinghamshire, UK). Nanoparticle tracking analysis was carried out using Malvern NanoSight NS500 (Malvern, UK), configured with a 405 nm laser and a high-sensitivity CMOS camera (OrcaFlash2.8, Hamamatsu C11440, NanoSight Ltd.). Videos were collected and analyzed using the NTA software (version 2.3, build 0025) with camera level and detection threshold set at 14 and 5, respectively. All analysis were carried out at a controlled temperature of 23 °C.

Protein samples and clinical serum samples

Fetal bovine serum (FBS), C-reactive protein (CRP), bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Sigma-Aldrich. α-Synuclein (α-Syn), Syntenin-1 (Synt-1) standards, anti-α-Syn, anti-Syn-1, anti-L1CAM, and anti-hemagglutinin (HA) antibodies were obtained from Abcam (Cambridge, UK). All protein samples were diluted in filtered PBS buffer solutions (pH 7.4).

Parkinson's disease (PD) and healthy controls (HC) were recruited and whole blood samples collected in compliance with the institutional guidelines and ethical approval at the Department of Neurology, Christian-Albrechts-University Kiel, Germany between 2013 and 2019. Full details of the Kiel-PD cohort were published previously.[1]

Preparation of antifouling pCBMA-coated MBs

The magnetic microbeads were prepared by a two-step approach comprising the formation of ferrihydrite/formaldehyde composite microbeads and subsequent

hydrothermal reduction of the ferrihydrite to magnetite.[2,3] Iron hydroxide was synthesized by hydrolysis of ferric chloride salt solution at room temperature as described previously.[3] Briefly, a total of 16 g of $NaHCO_3$ was slowly added to a 100 mL of ultrapure water in which 25 g of $FeCl_3 \cdot 6H_2O$ was dissolved. The mixture was stirred for 1 h to yield a reddish brown ferrihydrite solution, prior to the addition of 1.05 g of urea then a pH adjustment to 2.0 with 2 M nitric acid. This was followed by addition of 1.57 mL of aqueous formaldehyde (37 wt %) under stirring. After the addition was complete, the mixture was left without agitation at ambient temperature. Within 10 min, a yellowish gel is formed. The microspheres generated were allowed to age overnight, prior to collection by filtration and washing with Milli-Q water (18.2 MΩ, Millipore UK Ltd). Finally, the particle samples were suspended in 130 mL of 0.1 M sodium borohydride solution (pH 9.0), and the suspension transferred to an autoclave. The reaction was carried out at 80 °C for 2 h, during which the initially yellowish microspheres turn black, and can be readily magnetically extracted prior to a thorough wash with EtOH and Milli-Q water. These are then oven dried at 40 °C, and re-suspended in Milli-Q water at a concentration of 50 mg/mL.

The bead surfaces were functionalized with the bifunctional RAFT agent BisCTTC as follows: 1 mL of the Fe_3O_4 suspension was added to a 10 mL mixture of water/ethanol (3/7, v/v) under ultrasonication for 10 min at room temperature, followed by the addition of 10 mg of BisCTTC (0.044 mmol). A water/ethanol solvent was chosen to ensure dispersion of the magnetic beads and solubilization of BisCTTC. The mixture was left under magnetic stirring and a stream of nitrogen for 24 h. The final product of Fe_3O_4@ BisCTTC was separated and purified by magnetic collection and washed three times with ethanol and Milli-Q water.

In a final step, BisCTTC and 4,4'-Azobis (4-cyanovaleric acid) (ACVA) were used as monomer, free chain transfer agent (in solution phase) and initiator, respectively. Synthesis of pCBMA@Fe_3O_4 was performed through a standard RAFT polymerization procedure.[4,5] Typically, 1 mL of the suspension containing Fe_3O_4@ BisCTTC beads was mixed with CBMA (360 mg, 1.568 mmol), ACVA (1.1 mg, 0.00392 mmol) and free CTA BisCTTC (3.55 mg, 0.01568 mmol) dissolved in 10 mL of ethanol/water (1:1). After the reaction mixture was purged with nitrogen for one hour, the glass flask was heated in an oil bath at 70 °C, and left for 8 hours with mechanical stirring under

nitrogen. The reaction was terminated by inserting the reaction flask in an ice bath followed by exposure to air (quenching). The final pCBMA@Fe_3O_4 bead product was magnetically separated and washed several times with ethanol and water.

Fabrication of immunobeads

The antifouling immunobeads were prepared by conjugation of anti-L1CAM Ab (ab20148, Abcam) to pCBMA@Fe_3O_4. Specifically, the carboxyl acid groups of the pCBMA@Fe_3O_4 beads (1 mg/mL) were activated with 50 mg/mL of EDC/NHS in MES buffer[6] and then reacted with 8 µg/mL (final concentration) of anti-L1CAM or CD9 antibody at room temperature for 1.5 h. After washing with PBS using a magnet, the beads were mixed in 1 mL of PBS containing 5 mg/mL BSA (to quench any remaining activated sites and backfill any residual space), for 30 min at room temperature. The immunobeads were collected magnetically and stored at 4 °C until further use. All such immunobeads were prepared and consumed in the same day.

Fourier transform infrared- attenuated total reflectance (FTIR-ATR)

An appropriate amount of the prepared pCBMA magnetic beads were washed with ethanol and Milli-Q water and dried at 50 °C prior to examination. CBMA monomer and uncoated Fe_3O_4 magnetic beads were used as controls. All spectra were recorded between 4000–400 cm^{-1} with a Bruker Vertex 80 spectrometer equipped with mercury-cadmium-telluride (MCT) detector and an ATR-unit (DuraSamplIR II diamond ATR) at a resolution of 2 cm^{-1} and evaluated using OPUS 6.5 software.

Antifouling test for pCBMA beads

To test the antifouling performance of the pCBMA beads, 1 mg of the Ab-pCBMA@Fe_3O_4 or 1 mg of pCBMA@Fe_3O_4 (uncoated Fe_3O_4 beads were used as control) were added separately into 10 mg/mL BSA solution and incubated for 1 h at room temperature. Supernatant containing unbound protein were collected, subject to the bicinchoninic acid (BCA) test and adsorbed protein determined from:

Adsorbed amount = Input amount – Unbound amount in the supernatant

To evaluate the nonspecific adsorption level of free α-Synuclein to the immunobeads, 1 mg pCBMA magnetic beads coated with antiL1CAM antibody (anti-HA antibody or no antibody as controls), were added to 500 $\boldsymbol{\mu}$L PBS containing 20 ng/mL α-synuclein standard protein (i.e. a concentration that reflects clinically relevant levels of free α-synuclein in blood). The mixtures were gently shaking overnight at 4°C. After incubation, the supernatant fraction were collected using magnetic rack. Control beads (commercial carboxylate magnetic beads) with same experimental setting were carried out in parallel.

The adsorbed amount of α-synuclein onto the beads were quantified using the ECL kit using the following equation:

Adsorbed amount = Input amount – Unbound amount in the supernatant

Zeta potential

The surface zeta potential analysis was performed with uncoated Fe_3O_4 beads and pCBMA-coated MBs (ca. 1 mg/mL) in PBS (10 mM, pH = 7.4) on a Malvern Zetasizer Nano with a 532 nm laser as the light source.

Exosome isolation

For exosome isolation a 3-step sequential spin (300 g for 10 min, 2000 g for 20 min, and 10,000 g for 30 min) was used to remove cellular debris, protein aggregates and fatty material from the serum. An appropriate amount of supernatant (0.5 mL for commercial ECL plate and 0.1 mL for EIS sensor), *i.e.* pre-cleared serum, was transferred to protein low-binding tubes (Eppendorf) for immunocapture using anti-L1CAM antibodies pre-conjugated to pCBMA beads that were generated to reduce non-specific adsorption. The immunobeads were incubated at 4 ˚C overnight on a rotating mixer and bead-exosomes complexes were collected by magnetic separation and washed successively with 0.05 % Tween-20 in PBS (PBST) and PBS. For exosomal protein quantification the isolated exosomes were lysed in lysis buffer containing 1 % triton X-100 in PBS with 4% protease inhibitors (50 µL for commercial ECL plate and 10 µL for EIS sensor) for 15 min at room temperature for exosomal protein quantification.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to examine the shape and

morphology of the captured exosomes eluted from pCBMA beads. Specifically, the captured EVs on MBs were eluted by adding 20 µL of Glycine solution (pH 2.9) and the pH was adjusted back to neutral quickly with 20 µL of Tris solution (pH 9.5). 10 µl of resultant eluent samples was applied to freshly glow discharged carbon formvar 300 mesh copper grids for 2 mins, blotted with filter paper and stained with 2 % uranyl acetate (aqueous) for 10 s, then blotted and air dried. Grids were imaged with a TEM operated at 120 kV using a Gatan OneView CMOS camera.

Scanning electron microscopy

Immunocaptured exosomes on the pCBMA beads were fixed in 2 % glutaraldehyde on clean silicon wafer and washed twice with PBS. After natural evaporation, the samples were coated with around 5 nm platinum using a sputter coater (Cressington) and imaged with a scanning electron microscope at 5 kV (JEOL 6010LV).

Western blot

Western blot was used to characterize the transmembrane and internal proteins from immunocaptured exosomes. Exosomes captured by anti-L1CAM immunobeads (or anti-CD9 as positive control targeting generic exosomes and anti-HA immunobeads as negative control) were lysed in LDS buffer (Thermo Fisher) and resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto polyvinylidene fluoride membranes (PVDF, Invitrogen) and immunoblotted with antibodies against Synt-1 (ab133267, Abcam), CD9 (CBL162, Millipore), and L1CAM (ab80832, Abcam). All antibodies were used at 1:1,000 dilution. Following incubation with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare) (1:10,000 dilution), chemiluminescence was used for immunodetection (ChemiDoc, Bio-Rad).

Commercial electrochemiluminescence detection

Electrochemiluminescence (ECL) detection was performed in 96-well Meso Scale Discovery (MSD) U-Plex plates following the manufacturer instruction. Two unique linkers for the selected capture antibodies (anti-Synt-1, anti-α-synuclein) were used according to the manufacturer's protocol. Immunocaptured exosome lysates or

standards solution (50 µL) were loaded and incubated at room temperaure for 1h. After three washes, detection antibodies with Sulfo-TAG-labels were incubated for 1 hour. Following washes by wash buffer (from Meso Scale Discovery) and the addition of MSD Read buffer (from Meso Scale Discovery) the plates were read using the MSD-ECL platform (QuickPlex SQ 120). Data were analysed with the MSD Discovery Workbench 3.0 Data Analysis Toolbox. Antibody pairs for α-synuclein (preconjugated with biotin and ruthenium tag, provided by Meso Scale Discovery) were provided by MSD. Additive-free anti-Synt-1 goat polyclonal antibody (PAB7132, Abnova) and anti-Synt-1 rabbit monoclonal antibody (ab236071, Abcam) were conjugated with biotin and ruthenium and used as capture and detection antibodies, respectively.

Exosome capture efficiency

To evaluate the exosome capture efficiency using the immunobeads, anti-CD9 antibody modified pCBMA@Fe_3O_4 MBs were prepared following the same procedure of ‘‘**Fabrication of immunobeads**” on page S-5 in the supporting information. Immunobeads (0.2 mg) were mixed with 100 µL pre-cleared serum to allow incubation at 4 °C overnight. After incubation, the supernatants were collected with the aid of an external magnetic rack. The exosome concentration in the input serum and supernatants were then measured using a nanoparticle tracking analysis of particle fractions spanning 40 to 140 nm (i.e. typical size of exosomes).

The capture efficiency was measured using following equation,

(Input (CD9+ exosomes) – Unbound amount)/ Input (CD9+ exosomes) × 100%

= (Total input amount×75%[*] – Unbound amount)/ Total input amount×75%) × 100%

= (2.66-0.51)/2.66 ×100%

= 80.8 %

Note: [*] CD9+ exosomes constitute about 75 % of total exosome population[7]

Fabrication of receptor interface and EIS detection

Au disk electrodes (3.0 mm in diameter, purchased from BASi®, USA) were mechanically polished with 1.0 µm, 0.3 µm and 0.05 µm alumina slurry, respectively. The electrodes were ultrasonicated in ethanol for 10 min, and immersed in piranha (v/v 3:1, H_2SO_4:H_2O_2, *Caution: piranha reacts extremely aggressively with organic materials. Extreme caution needed!*) for 10 min. After rinsing with Milli-Q water and dried with nitrogen, the electrodes were immersed in 0.5 M KOH aqueous solution for 100 cycles of cyclic voltammetry scans (from−1.7 to −0.7 V). They were then electrochemically cycled in 0.5 M H_2SO_4 from -0.15V to 1.35V vs an Ag wire reference electrode at 0.1 V/s until the height and shape of anodic and cathodic peaks were constant.

Mixed SAMs of 3-MPA and 2-MU were generated by immersion of clean gold disk electrodes in 50 mM 3-MPA and 10 mM 2-MU solution overnight at room temperature in the dark. The electrodes were rinsed with ethanol to remove physically adsorbed molecules and then dried in an argon stream. The terminal carboxyl groups of 3-MPA were then activated with 0.4 M EDC/NHS solution for 30 min,[8] and washed carefully with PBS. 10 µL of antibody solution with an optimized concentration of 100 µg/mL was then incubated on the electrode for 1 h, and the surface was then blocked with FBS solution for 30 min to deactivate any residual carboxylic groups. The stability of antibody-modified electrode was tested by repetitive incubating in PBS for 20 mins and subsequent EIS assessments in 5 mM of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$. Afterwards, 10 µL of α-Syn, Synt-1 spiked into 10% human serum or exosomes lysate (obtained by adding 1 % triton X-100 in PBS with 4 % protease inhibitors to the exosomes-beads composite at room temperature for 15 min) was then incubated on the electrode for an optimized incubation time of 20 mins (Figure S3 B), and washed with PBS solution. Selectivity analyses (Figure S3) were conducted by incubating sensor electrodes with 10^{-3} g/mL of CRP, 10^{-3} g/mL of α-Syn, or 10^{-3} g/mL of BSA for 20 mins prior to washing with PBS solution. EIS measurements were recorded with a PalmSens electrochemical workstation with a standard three electrode configuration, and they were conducted in 5 mM of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ in PBS solution. All measurements were carried out with setting fixed at amplitude 0.01 V and frequencies ranging from 100 kHz to 100 mHz. R_{ct} upon addition of antibody ($R_{ct\text{-}antibody}$) and

antigen ($R_{ct\text{-}antigen}$) were calculated from the fitting of equivalent circuit diagram. The relative response are determined from:

Relative response = $R_{ct\text{-}antigen} - R_{ct\text{-}antibody}$.

Statistical analysis of patient samples were through a standard Student's t-test.

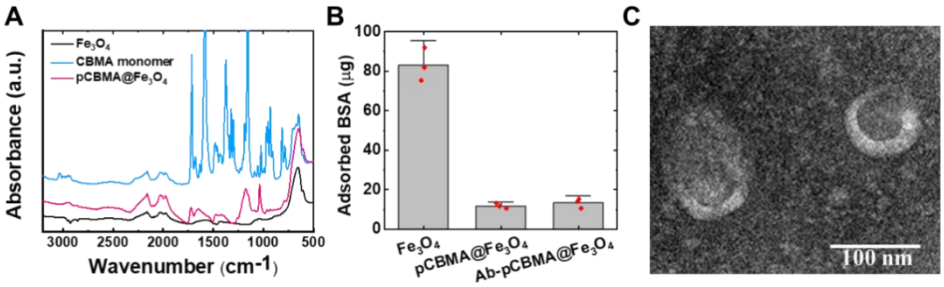

Figure. S1 (A) FTIR-ATR spectrum of Fe_3O_4, pCBMA@Fe_3O_4 and CBMA monomer. (B) Histogram depicts a quantitative assessment of adsorbed BSA on different MBs surfaces (1 mg beads input). The error bar represents the standard deviation of three distinct collected experimental data sets. (C) TEM image of typical captured and eluted vesicles confirming their double-membrane organization structure and size (~100nm).

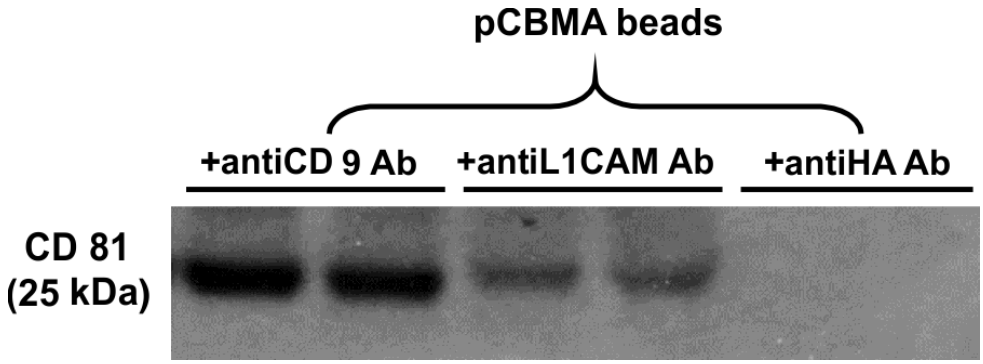

Figure S2 Immunoblot (CD 81) of immunocaptured exosomes from serum using anti-CD9 and anti-L1CAM targeting generic exosomes and neuronal exosomes, respectively. pCBMA beads without antibody were used as control. The ratio of L1CAM+ (neuronal exosomes)/CD9+(generic exosomes) was calculated to be ~11 % based on a comparative analysis of immunoblot intensities of CD81 (generic exosomal transmembrane protein) bands. With anti-HA (control) antibody modified beads, no

CD81(surface biomarker of exosomes) was detected. This indicates the selective isolation of neuronal exosomes at anti-L1CAM polymer beads.

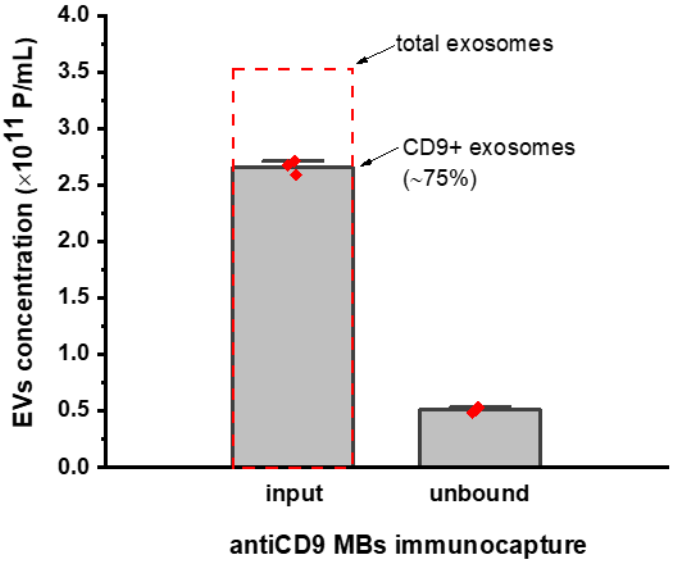

Figure S3. Histogram of the capture efficiency evaluation measured by nanoparticle tracking analysis using anti-CD9 modified pCBMA beads. The error bar presents the standard deviation of three individually collected experimental data.

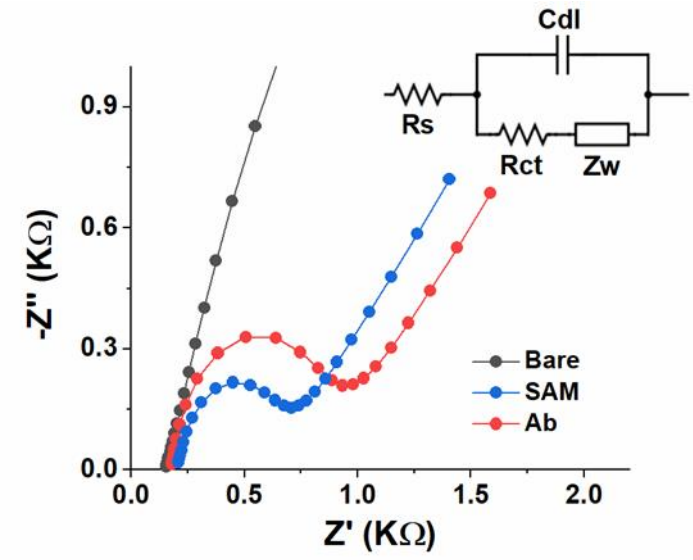

Figure S4. EIS characterization of bare gold working electrode (black curve), mixed SAM modified gold working electrode (blue curve), and antibody modified gold working electrode (red curve). Inset shows the equivalent circuit diagram for data fitting of the impedance data. R_s = solution resistance, R_{ct} = charge transfer resistance, C_{dl} is the double layer capacitance, and Z_w = warburg impedance.

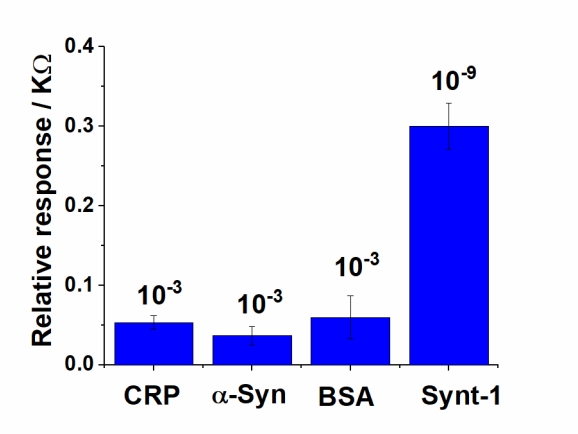

Figure S5. Relative responses of anti-Synteinin-1 modified sensor to 10^{-3} g/mL of CRP, 10^{-3} g/mL of α-Syn, 10^{-3} g/mL of BSA and 10^{-9} g/mL Synt-1. The error bars were calculated from 9 measurements: triplicate repeats across three experiments using 3 independent working electrodes.

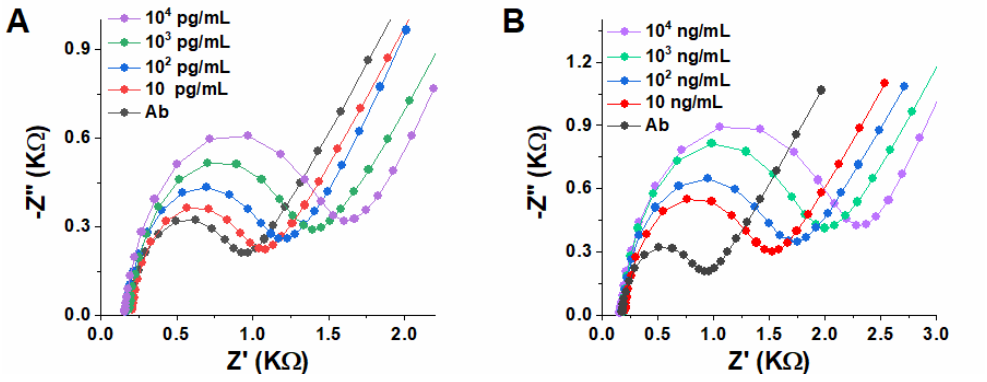

Figure S6. Nyquist curves of (A) anti-α-Syn modified working electrode to α-Syn spiked into 10% human serum with concentrations of 10 pg/mL (red curve), 10^2 pg/mL (blue curve), 10^3 pg/mL (green curve), and 10^4 pg/mL (purple curve). (B) anti-Synteinin-1 modified working electrode to Synt-1 spiked into 10% human serum with concentrations of 10 ng/mL (red curve), 10^2 ng/mL (blue curve), 10^3 ng/mL (green curve), and 10^4 ng/mL (purple curve).

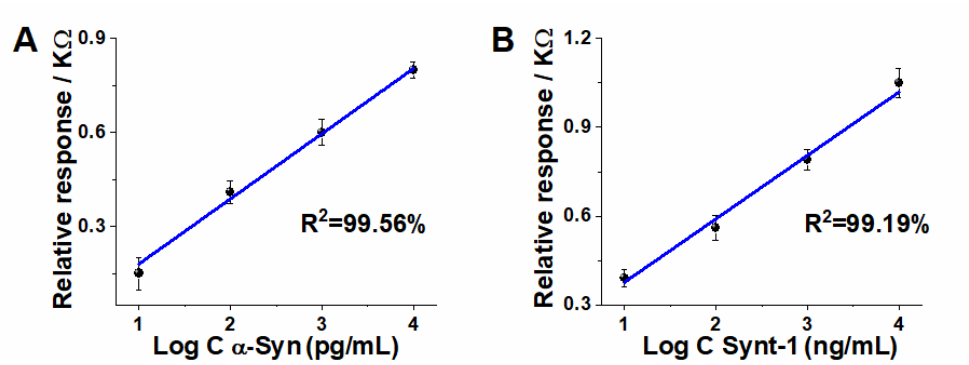

Figure S7. Impedimetric calibration curves for (A) α-Syn spiked into 10% human serum with a dynamic range from 10 to 10^4 pg/mL, and (B) Synt-1 spiked into 10% human serum in a concentration range of 10 to 10^4 ng/mL. The error bars were calculated from 9 measurements: triplicate repeats across three experiments using 3 independent working electrodes.

Figure S8 shows a fitting of the synuclein data as used in Figure S7 to a Langmuir−Freundlich isotherm:

$$\theta = \frac{\mathrm{K} \times [\alpha - \mathrm{Syn\ or\ Synt} - 1]^{\mathrm{n}}}{1 + \mathrm{K} \times [\alpha - \mathrm{Syn\ or\ Synt} - 1]^{\mathrm{n}}}$$

where θ is the fractional occupancy of receptive sites and K is the binding constant. The fits are excellent and resolve θ as 5% for α-Syn at its LOD concentration (0.3 pg/mL).

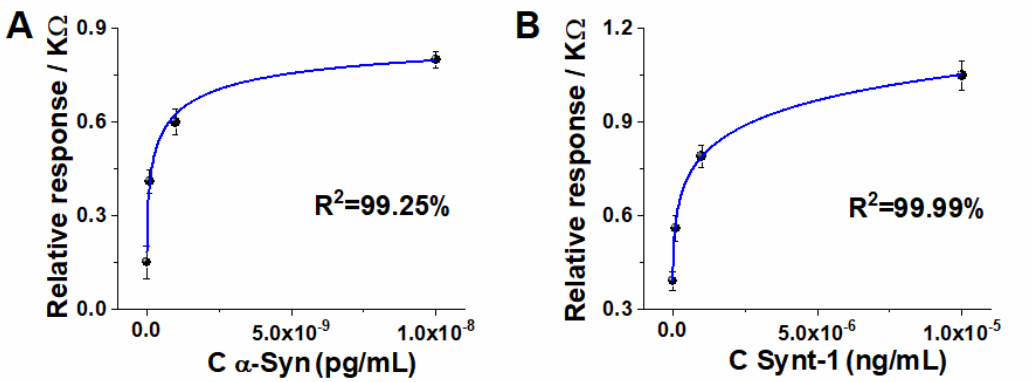

Figure S8. Relative response of anti-Synt-1 modified sensor in the presence increasing concentration of (A) α-Syn and (B) Synt-1. The error bars were calculated from 9 measurements: triplicate repeats across three experiments using 3 independent working electrodes.

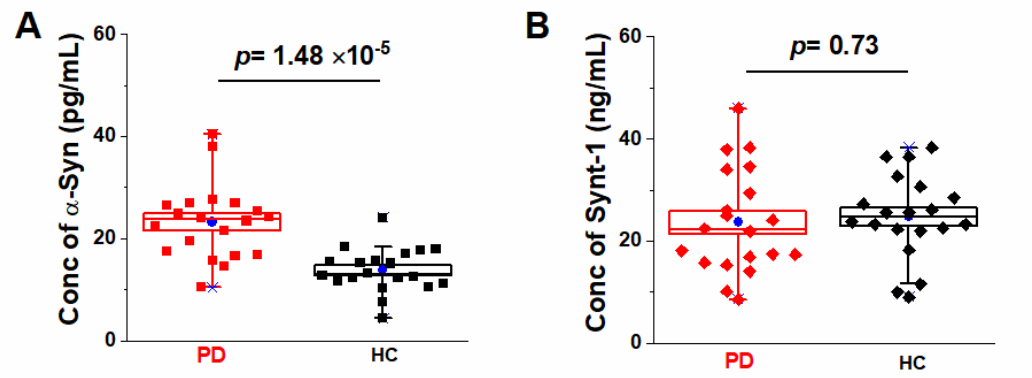

Figure S9. (A) Box plots for exosomal α-Syn detected by electrochemiluminescence kit (sample number n = 40). (B) Box plots for exosomal Synt-1 detected by electrochemiluminescence kit. In the box plots, the lower and upper boundaries indicate the 25th and 75th percentiles respectively. The line within the box marks the median, and the blue circle within the box marks the mean. Diamonds represent individual patient sample data points.

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