On-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry for the analysis of blood α-synuclein

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KEYWORDS: aptamer / capillary electrophoresis / in-line solid-phase extraction / mass spectrometry / on-line solid-phase extraction / α-synuclein

ABSTRACT

In this paper, an on-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry method is described for the purification, preconcentration, separation, and characterization of α-synuclein (α-syn) in blood at the intact protein level. A single-stranded DNA aptamer is used to bind with high affinity and selectivity α-syn, which is a major component of Lewy bodies, the typical aggregated protein deposits found in Parkinson's disease (PD). Under the conditions optimized with recombinant α-syn, repeatability (2.1 and 5.4% percent relative standard deviation for migration times and peak areas, respectively) and microcartridge lifetime (around 20 analyses/microcartridge) were good, the method was linear between 0.5 and 10 $\mu g \cdot mL^{-1}$ and limit of detection was 0.2 $\mu g \cdot mL^{-1}$ (100 times lower than by CE-MS, 20 $\mu g \cdot mL^{-1}$). The method was subsequently applied to the analysis of endogenous α-syn from red blood cells lysate of healthy controls and PD patients.

Capillary electrophoresis-mass spectrometry (CE-MS) is regarded nowadays as a powerful technique for the highly efficient separation and characterization of biomolecules, including peptides, protein isoforms and post-translational modifications (PTMs) or protein complexes[1–4]. However, as in many other microscale separation techniques, the small sample volume injected for optimum separation (typically 1-2% of the capillary volume) compromises the concentration sensitivity for most analytes and is very often a limitation that hinders a more widespread application[5–9]. To improve the limits of detection (LODs), the use of more selective and sensitive mass spectrometers is in many cases not satisfactory enough. Therefore, for the on-line preconcentration of the target analytes after the injection of a large volume of sample, CE-MS has been often combined with different electrophoretic[5,6] and chromatographic[6–9] techniques. Within the chromatographic preconcentration techniques, on-line solid-phase extraction capillary electrophoresis (SPE-CE) is widely recognized as an excellent option to preconcentrate and clean up the target analytes, minimizing sample handling and increasing analysis throughput. In the most widely used SPE-CE configuration, a microcartridge with an appropriate sorbent to selectively retain the target analyte is integrated in-line near the entrance of the separation capillary and no valves are necessary for the operation. After loading a large volume of sample (~50-100 μL), the capillary is rinsed to eliminate non-retained molecules and filled with background electrolyte (BGE). Then, the analyte is eluted and preconcentrated in a small volume of an appropriate solution (~25-50 nL) before the separation and the detection[8,9].

Nowadays, the availability of a wide variety of commercial or lab-made sorbents has broadened the applicability of SPE-CE. SPE-CE has been explored using conventional chromatographic sorbents (e.g. C18 or HLB)[7–9], but there is an urgent need of high selective affinity sorbents to analyze complex samples such as biological

fluids[9–15]. Immunoaffinity (IA) sorbents prepared by immobilization of antibodies or antibody fragments are for many authors the gold standard within the high selective sorbents and can provide excellent sample clean-up and extraction efficiency. However, the applicability of IA-SPE-CE-MS is limited by several constraints related to the conditions needed to guarantee the antibody stability. On-line MS detection is negatively affected by the presence of salts due to ionization suppression and requires the use of volatile solutions and BGEs to prevent salt build-up. The typically acidic BGEs used in CE-MS in positive electrospray ionization (ESI+) mode (e.g. from 0.1 to 1 M of acetic acid) may cause antibody denaturation, therefore, compromising IA sorbent stability. This issue is usually solved using neutral BGEs (e.g. 10 mM of ammonium acetate), but ionization efficiency is in general lower under these conditions. As an interesting alternative to antibodies, the use of aptamer-based sorbents has been proposed for sample preparation[16]. The most common aptamers are single-stranded oligonucleotides that are able to bind to a target molecule with high-affinity and selectivity. These features enable their application in diagnostics, imaging, therapeutics, targeted delivery, and biosensing[17–20]. In general, aptamers are isolated in vitro via systematic evolution of ligands by exponential enrichment (SELEX), an iterative cycle of selection and amplification steps that enriches high-affinity and selective aptamers from a large combinatorial library[20,21]. Aptamers have been generated for a wide variety of targets, ranging from simple inorganic molecules to proteins and whole cells[20,21]. Aptamers are chemically synthesized, which eliminates the requirement of animals or cells and the possible batch-to-batch variations associated with antibodies, and are significantly faster and cheaper to produce[20]. Other interesting advantages of aptamers are robustness, thermal stability, tolerance to wide ranges of pH and salt concentration. Furthermore, they can be chemically modified at either 3’ or 5’-terminus to incorporate

various functional groups and spacer arms to facilitate the covalent immobilization with an appropriate orientation and minimal steric hindrance on a solid support[16,22].

The use of aptamer affinity (AA) sorbents has been previously described for off-line solid phase microextraction (SPME)[23], on-chip SPE[24], and on-line SPE-nanoLC[22]. Recently, Marechal et al.[14] described an aptamer silica-based monolithic microcartridge for the analysis of ochratoxin A in standards, beer and wine by AA-SPE-CE with laser induced fluorescence (LIF) detection. So far, to the best of our knowledge, no other applications of AA-SPE-CE have been described.

In this paper, AA-SPE-CE-MS is described for the first time and a method for the analysis of α-synuclein (α-syn) in blood is developed. α-Syn is a 14 kDa protein, composed of 140 amino acids and numerous proteoforms, including PTMs as phosphorylation, ubiquitination, nitration, and acetylation[25–27]. It is present at high levels in the brain where it is mainly localized in presynaptic terminals of nerve cells[25], but it can be also found in biological fluids such as cerebrospinal fluid (CSF), blood and saliva[28,29]. The potential of certain α-syn proteoforms as biomarkers for early diagnosis and tracking progression of Parkinson's disease (PD), one of the most common neurodegenerative motor disorders, is continuously investigated. Phosphorylated α-syn at Ser-129 is a major component (90% of α-syn) in the aggregates of Lewy bodies (LB), which represent the morphological hallmark of PD[25]. In contrast, full-length *N*-terminal acetylated α-syn is the major proteoform in brain cytosol whereas phosphorylated α-syn involves only about 4% of normal α-syn[25]. α-Syn has been frequently analyzed in brain tissue or CSF[26,30]. However, more accessible biofluids, such as blood, are also being investigated[30,31], where it is still necessary to broaden the knowledge about the different proteoforms using novel, sensitive and selective analytical methods, as the one proposed in this study.

Experimental section

Materials and reagents

All the chemicals used in the preparation of background electrolytes (BGEs) and solutions were of analytical reagent grade or better. Acetic acid (HAc) (glacial), ammonium hydroxide (NH_4OH) (25%), formic acid (HFor) (99.0%), potassium chloride (99.0%), potassium dihydrogenphosphate (≥99.0%), sodium chloride (≥99.5%), and sodium hydrogenphosphate (≥99.0%), were purchased from Merck (Darmstadt, Germany). Acetonitrile (LC-MS), and ethanol (96%) were supplied by Panreac AppliChem (Barcelona, Spain). Ammonium acetate (NH_4Ac) (≥99.9%), and Tween® 20 were supplied by Sigma-Aldrich (Steinheim, Germany). Propan-2-ol (LC-MS) was purchased from Scharlau (Barcelona, Spain). Water (LC-MS grade) was supplied by Fisher Scientific (Loughborough, UK).

The DNA aptamer M5-15[32] modified with a C6 spacer arm terminated by 5'amino (M5-15-5', 66-mer, M_r = 20,690) and the masking DNA (T-SO508[33], an off-target sequence single-stranded DNA that tested negative for α-syn by AA-SPE-CE-MS and was already available in the laboratory, 24-mer, M_r = 7708), both purified by HPLC, were purchased from Integrated DNA Technologies (Coralville, IA, USA).

Magnetic beads (MBs) LOABeads™ AffiAmino of 45-165 µm diameter were purchased from Lab on a Bead (Uppsala, Sweden).

Electrolyte solutions, sheath liquid, protein standards and blood samples.

All the solutions were degassed for 10 min by sonication before use. The optimized BGE for the CE-MS and AA-SPE-CE-MS separation contained 100 mM HAc (pH 2.9). The BGE was passed through a 0.20 µm nylon filter (Macherey-Nagel, Düren, Germany). The sheath liquid solution consisted of a mixture of 60:40 (*v/v*)

propan-2-ol/water with 0.05% (*v/v*) of HFor and was delivered at a flow rate of 3.3 $\mu L \cdot min^{-1}$ by a KD Scientific 100 series infusion pump (Holliston, MA, USA).

Recombinant human α-syn expressed in *Escherichia coli* was purchased from Analytik Jena (Jena, Germany). The solution provided by the manufacturer (5000 $\mu g \cdot mL^{-1}$ in phosphate buffered saline (PBS)) was aliquoted and stored in a freezer at -20ºC. Aliquots were thawed before use and working standard solutions were prepared by dilution in water. These solutions were stored in the fridge at 5ºC when not in use.

Human blood samples from patients were provided by the Basque Biobank/Biodonostia Node (www.biobancovasco.org). Samples were processed following standard operation procedures with appropriate approval of the Ethical and Scientific Committees. Three healthy donor blood samples and three PD patient blood samples (one at stage III and two at stage IV of the disease) were analyzed. All the samples corresponded to males and females aged between 60 and 80 years.

Pretreatments of red blood cells lysates

Red blood cells (RBCs) lysates were prepared from blood as described in the Supporting Information[34].

The RBCs lysates were precipitated with ethanol-chloroform to deplete hemoglobin as described elsewhere[34], with some changes: 350 µL of cold ethanol and 200 µL of cold chloroform were added to 250 µL of RBCs lysate. The mixture was shaken for 5 min at 4ºC and centrifuged at 3000 g for 10 min at 4ºC. The supernatant was collected and low M_r compounds were removed with 10,000 M_r cut-off (MWCO) cellulose acetate centrifugal filters (Amicon Ultra-0.5, Millipore).

Thermo-enrichment to deplete the non-thermostable proteins was performed on the RBCs lysates as following[31]: 350 µL of RBCs lysate were heated at 90ºC for 10 min

in a thermoshaker. The mixture was centrifuged at 12000 g for 5 min at 4ºC and the supernatant (i.e. thermo-enriched (TE) RBCs lysate) was filtered using a 0.22 µm polyvinylidene difluoride centrifugal filter (Ultrafree-MC, Millipore, Bedford, MA, USA) at 12000 g for 5 min.

Apparatus

pH measurements were made with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain). Agitation was performed with a Vortex Genius 3 (Ika®, Staufen, Germany). Centrifugal filtration was carried out in a Mikro 220 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Incubations were carried out in a TS-100 thermoshaker (Biosan, Riga, Latvian Republic). A neodymium cube magnet (12 mm, N48) was supplied by Lab on a Bead.

CE-MS

Fused silica capillaries were supplied by Polymicro Technologies (Phoenix, AZ, USA). All CE-MS experiments were performed in a 7100 CE coupled with an orthogonal G1603A sheath-flow interface to a 6220 oa-TOF LC/MS spectrometer (Agilent Technologies, Waldbronn, Germany). ChemStation and MassHunter softwares (Agilent Technologies) were used for the CE and TOF mass spectrometer control, data acquisition and processing. The TOF mass spectrometer was operated in ESI+ mode and the optimized parameters are presented in the Supporting Information.

Separations were performed at 25°C in a 72 cm long (L_T) × 75 µm i.d. × 365 µm o.d. capillary. All capillary rinses were performed flushing at 930 mbar. For new capillaries or between workdays, the capillaries were flushed off-line with 1 M NaOH (15 or 5 min, respectively), water (15 or 10 min), and BGE (30 or 15 min) to avoid the

unnecessary contamination of the MS system. Samples were hydrodynamically injected at 50 mbar for 10 s (54 nL, i.e. 1.7% of the capillary, estimated using the Hagen–Poiseuille equation[35]), ,and a separation voltage of +25 kV (normal polarity, cathode in the outlet) was applied. The autosampler was kept at 10°C using an external water bath (Minichiller 300, Peter Huber Kältemaschinenbau AG, Offenburg, Germany). Between runs, the capillary was conditioned flushing with water (2 min) and BGE (2 min).

AA-SPE-CE-MS

AA-MBs were prepared following the manufacturer recommendations. First, a 200 µL aliquot of MBs solution was vortexed and the supernatant was removed after magnetic separation, using a cube magnet to sediment the particles (20 µL of sedimented MBs). The MBs were washed using 200 µL of PBS with 0.1% Tween® 20 (PBS-T), the supernatant was removed by magnetic separation and the MBs resuspended with the same volume of PBS-T. Ten µL of activation buffer was added and the MBs were moderately shaken for 15 min at room temperature. The supernatant was removed by magnetic separation, the MBs were washed with 200 µL of PBS-T and resuspended with 150 µL of PBS-T. Fifty µL of the DNA aptamer M5-15-5'amino dissolved in PBS (100 $\mu mol \cdot L^{-1}$) was then added to the MBs suspension. The mixture was moderately shaken for 40 min at room temperature. The supernatant was removed and the AA-MBs were subsequently washed three times with 200 µL of PBS and resuspended with the same volume of PBS. The remaining reactive groups on AA-MBs were blocked adding 20 µL of blocking buffer (50% (*v/v*) ethanolamine in PBS) and the mixture was moderately shaken for 45 min at room temperature. Finally, the supernatant was removed and the AA-MBs were subsequently washed three times with

200 µL of PBS. The AA-MBs were stored in PBS with 20% (*v/v*) ethanol at 4ºC when not in use.

Construction of fritless particle-packed microcartridges for AA-SPE-CE-MS was carried out as described elsewhere, taking advantage of the average larger size of the sorbent particles compared to the inner diameter of the separation capillary[9,13,36]. The microcartridge (0.9 cm L_T × 250 µm i.d. × 365 µm o.d. capillary) was completely filled by vacuum with AA-MB sorbent and connected with plastic sleeves to two capillary fragments (7.5 cm L_T × 75 µm i.d. × 365 µm o.d. (inlet) and 64.5 cm L_T × 75 µm i.d. × 365 µm o.d. (outlet)), which were conditioned before beginning the construction.

Under the optimized conditions, AA-SPE-CE-MS capillaries were first conditioned flushing with BGE at 930 mbar for 2 min. Samples were introduced at 930 mbar for 5 min (30 µL[35]). A final flush with BGE for 2 min eliminated nonretained molecules and filled the capillary before the electrophoretic separation. All these steps were performed with the nebulizer gas and the ESI capillary voltage switched off to prevent the entrance of contaminants into the MS. Then, both were switched on and a small volume of eluent with 100 mM NH_4OH (pH 11.2) was injected at 50 mbar for 20 s (100 nL[35]). For a rapid and repeatable protein elution, the small plug of eluent was pushed with BGE at 50 mbar for 100 s, before applying the separation voltage (+25 kV) and a small pressure (25 mbar) to compensate for the microcartridge counter-pressure. Between consecutive runs, to avoid carry-over, the capillary was flushed with water for 1 min, eluent was injected at 50 mbar for 40s, and the capillary was flushed again with water for 1 min. All experiments were performed at 25ºC.

Quality parameters

The details regarding the limit of detection (LOD), limit of quantification (LOQ), repeatability of migration time and peak area, linearity, and microcartridge lifetime in CE-MS and AA-SPE-CE-MS are given in the Supporting Information.

LC-Orbitrap-MS/MS

The details about TE RBCs lysate bottom-up proteomics workflow including LC-Orbitrap-MS/MS analysis are given in the Supporting Information.

Results and discussion

CE-MS

In general, the best results for the analysis of intact proteins by CE-MS in ESI+ are obtained using acidic volatile BGEs and sheath liquids because protein ionization is maximized and the best sensitivity is achieved. Different conditions were tested for the analysis of recombinant human α-syn (i.e. 50 mM HAc: 50 mM HFor (pH 2.3); 100 mM HAc (pH 2.9) or 10 mM NH_4Ac (pH 7.0, 8.0 or 9.0) as BGEs combined with 60:40 (*v/v*) propan-2-ol/water with 0.05 or 0.25% (*v/v*) of HFor as sheath liquids). The best results for the analysis of α-syn were obtained with a BGE of 100 mM HAc (pH 2.9) and a sheath liquid of 60:40 (*v/v*) propan-2-ol/water with 0.05% (*v/v*) of HFor. As an example, Figure 1 shows the extracted ion electropherogram (EIE) (A), mass spectrum (B) and deconvoluted mass spectrum (C) for the CE-MS analysis of a 100 $\mu g \cdot mL^{-1}$ standard solution of recombinant human α-syn in the optimized conditions. The only detected proteoform was free α-syn because the recombinant human α-syn expressed in *E. coli* was not supposed to undergo post-translational modifications (PTMs) during bacterial synthesis (the minor peaks in the deconvoluted mass spectrum of Figure 1-C

are mostly due to Na^+ and K+ adducts to the ion species of the mass spectrum of Figure 1-B). Table S-1 shows the theoretical average M_r of free α-syn and the relative error (E_r) for the experimental deconvoluted average M_r. Mass accuracy was good (E_r<20 ppm). Under the optimized conditions, consecutive analyses of the α-syn standard were repeatable in terms of migration time and peak area (%*RSD* (n=3) were 0.9 and 5.6% at 100 $\mu g \cdot mL^{-1}$). The LOD was 20 $\mu g \cdot mL^{-1}$, better than the 50 $\mu g \cdot mL^{-1}$ LOD by CE-MS using a BGE of 10 mM NH_4Ac (pH 7.0) and a sheath liquid of 60:40 (*v/v*) propan-2-ol/water with 0.25% (*v/v*) of HFor. These latter conditions are typically required to analyze proteins by IA-SPE-CE-MS[12,13].

AA-SPE-CE-MS optimization

The DNA aptamer M5-15 was selected to prepare the aptamer affinity (AA) sorbent because it has been described to selectively bind with high affinity to α-syn monomer[32]. In contrast to our previous studies by IA-SPE-CE-MS[12,13], it was observed that in AA-SPE-CE-MS the AA sorbent was stable even using acidic BGEs. Sensitivity and repeatability were investigated with different combinations of BGE and sheath liquid (see below), using a basic volatile eluent of 100 mM NH_4OH (pH 11.2)[12,13]. Using a sheath liquid of 60:40 (*v/v*) propan-2-ol/water with 0.05% (*v/v*) of HFor, a BGE of 100 mM HAc (pH 2.9) allowed obtaining similar peak areas to a BGE of 50 mM HAc: 50 mM HFor (pH 2.3). However, in the second case, a slight decrease of peak areas was detected after consecutive injections, probably because of AA sorbent deterioration due to the lower pH value. Using the basic eluent, the acidic BGEs provided higher sensitivity than the BGEs of 10 mM NH_4Ac at pH 7.0, 8.0 or 9.0, even with a sheath liquid of 60:40 (*v/v*) propan-2-ol/water with an increased amount of HFor (i.e. 0.25% (*v/v*) of HFor). To confirm that no analyte was eluted under acidic conditions, BGEs of

10 mM NH_4Ac and an eluent of 100 mM HAc (pH 2.9) were tested and no α-syn was detected. Therefore, the BGE of 100 mM HAc (pH 2.9) was selected for the rest of the experiments.

The investigation of the volatile eluents was extended, testing different hydroorganic mixtures in the presence or absence of 100 mM NH_4OH. First, acetonitrile:water mixtures at 40% and 80% (*v/v*) were tested to disrupt analyte-aptamer interaction[14,23]. However, these acetonitrile:water eluents were rapidly discarded because electropherograms and mass spectra were extremely poor, probably due to sorbent deterioration during the elution. Results with 60% (*v/v*) MeOH in the presence or absence of 100 mM NH_4OH were neither satisfactory as shown in Figure 2A. The best sensitivity and repeatability of peak areas and migration times were obtained with the aqueous basic eluent of 100 mM NH_4OH (pH 11.2). A higher concentration than 100 mM of NH_4OH in the aqueous eluent was not tested to prevent aptamer denaturation and expand the sorbent lifetime. The volume of the eluent plug was investigated injecting the eluent at 50 mbar for 10, 20 and 40 s (50, 100 and 200 nL[35]). A higher amount of α-syn was detected injecting the eluent 20 s instead of 10 s. However, for the 40 s eluent injection, the analyte peak broadened, and peak area decreased. Between consecutive runs of a 10 $\mu g \cdot mL^{-1}$ α-syn standard solution, a small amount of α-syn was detected as carry-over when only rinsing with water between injections. Therefore, to prevent carry-over, the capillary was rinsed with water, a small plug of eluent, and again water between injections.

With regard to the sample loading, the standard solutions were prepared in water because lower peak areas were observed when using PBS, probably due to the smaller binding efficiency in a salty environment. The sample loading time was studied introducing a 1 $\mu g \cdot mL^{-1}$ α-syn standard solution at 930 mbar from 3 to 15 min. As can

be seen in Figure 2B, the maximum amount of α-syn was detected at around 5 min. When loading for a longer time, the sample breakthrough volume was exceeded and the α-syn washed away was higher than the amount retained, causing a significant decrease of peak area. Therefore, to reduce the total analysis time and to obtain the highest recoveries, a sample loading time of 5 min was selected for the rest of the experiments.

Under the optimized conditions, consecutive analyses of the standard were repeatable in terms of migration time and peak area. At 1 μg·mL^{-1}, the %*RSD*s (n=3) were 2.1 and 5.4%, respectively, similar to the values in CE-MS. As can be seen in Figure 2C, the method was satisfactorily linear (R^2>0.994) between 0.5 and 10 μg·mL^{-1}. The LOQ was 0.5 μg·mL^{-1} and the LOD was 0.2 μg·mL^{-1}, which was an improvement of about 100 times compared to the CE-MS method. The lifetime of the microcartridges was around 20 analyses (at 1 μg·mL^{-1}). As an example, Figure 1A shows the AA-SPE-CE-MS analysis of a 1 μg·mL^{-1} α-syn standard. Compared to CE-MS (Figures 1B, 1C and Table S-1), the mass spectrum and mass accuracy for experimental deconvoluted average M_r of free α-syn was similar (data not shown).

Analysis of α-syn in blood samples

The AA-SPE-CE-MS method optimized with standards was applied to the analysis of blood. The concentration of α-syn has been shown to be higher in blood than in cerebrospinal fluid (CSF) and, furthermore, drawing blood is less invasive than lumbar puncture to obtain CSF from patients[29,30]. More than 99% of the α-syn in human blood resides in the red blood cells (RBCs)[37]. *N*-terminal acetylation of proteins is a common occurrence, especially for those proteins initiated in Met residue, and this PTM of α-syn is the most abundant in blood and also in brain cytosol[26].

A healthy control RBCs lysate was prepared, passed through a 0.20 µm nylon filter and analyzed by CE-MS and AA-SPE-CE-MS. In both cases, was detected only hemoglobin (Hb) (Figure S-1 and Table S-2), which constitutes around 95% of the protein content in the RBCs[38]. Therefore, to analyze low abundant proteins (i.e. endogenous α-syn) it was necessary to study different pretreatments to remove the most abundant proteins from the RBCs lysate, reducing sample complexity and the dynamic concentration range of the proteins. Ethanol-chloroform extraction to deplete Hb from the RBCs lysate was tested as in our previous work to purify superoxide dismutase from RBCs lysates[34]. With this pretreatment, Hb quantitatively precipitated from the RBCs lysate because Hb was not detected in the analysis of both the organic and aqueous phases by CE-MS. However, both phases contained other high abundant proteins that interfered in α-syn detection. In the analysis by CE-MS and AA-SPE-CE-MS of both phases, ubiquitin and *N*-acetylated carbonic anhydrase 1 (CA-1) were detected but not α-syn (Figure S-2 and Table S-2). These results were consistent with the fact that ethanol-chloroform extraction has been also historically used to achieve quantitative removal of Hb in purification of carbonic anhydrases[39]. However, an alternative purification method was necessary for α-syn.

Taking into account that α-syn is a thermostable protein, and the solubility and the PTMs pattern of α-syn is not supposed to be altered upon heating[27,31,40], the RBCs lysate was thermally treated to deplete non-thermostable proteins. With this pretreatment, both Hb and CA-1 were removed from the RBCs lysate but endogenous α-syn was not detected by CE-MS. Only ubiquitin and apolipoprotein A-I, which were highly abundant in the RBCs[38,41], were detected (Figure S-3 and Table S-2). With regard to the analysis of the TE RBCs lysate by AA-SPE-CE-MS (Figure 3), it was detected at around 14 min (Figure 3A) a protein with a deconvoluted M_r of 14,502.14

(Figure 3C), which is a highly consistent value with the expected average M_r for *N*-acetylated α-syn (M_r 14,502.06; Table S-1). In order to check if *N*-terminal acetylation of endogenous α-syn significantly affected the electrophoretic mobility of the protein and was causing the increase on migration time compared to the standard in water (~10 min increase), a TE RBCs lysate sample spiked with 0.3 $\mu g \cdot mL^{-1}$ of free α-syn standard was analyzed (recovery for free α-syn was 86%, by comparison of the AA-SPE-CE-MS analyses for the standard and the spiked sample at 0.3 $\mu g \cdot mL^{-1}$). As can be observed in Figure 4A, free α-syn and *N*-acetylated α-syn comigrated. However, the excellent mass accuracy and resolving power of the mass spectrometer allowed to unambiguously identify both proteoforms in the deconvoluted mass spectrum of Figure 4-C ($\Delta M_r = +42$) and to obtain separate EIEs (Figure 4A). With regard to the migration time increase detected in TE RBCs lysates, it was probably due to the modification of the inner wall of the separation capillary induced during sample loading by the non-retained components of the complex sample matrix. This modification happened after the first analysis with a new microcartridge and was permanent, because repeatability was high (see the quality parameters below). Another proof of this permanent modification was that free α-syn was also detected at this increased migration time when a standard α-syn solution was analyzed after a TE RBCs lysate sample.

As can be also observed in Figures 3 and 4, *N*-acetylated α-syn slightly comigrated with ubiquitin, which was also retained by the AA sorbent. In addition to ubiquitin, very small amounts of apolipoprotein A-I were detected (Figure S-4). Several experiments were made to investigate non-specific adsorption on the AA sorbent. It is worth highlighting that compared to the typical aptamer- or antibody-based biosensors or bioassays, a great advantage of AA-SPE-CE-MS is that the electrophoretic separation and the selectivity of the MS detection prevent the possibility of a false positive or an

erroneous quantification of the target protein in the presence of non-specific adsorption. A microcartridge containing blank sorbent (i.e. activated and endcapped MBs, without aptamer) was tested by SPE-CE-MS. When loading a 1 $\mu g \cdot mL^{-1}$ α-syn standard solution a very small amount of α-syn was detected (6.4% compared to the AA sorbent). This suggested that non-specific adsorption of α-syn was very limited because it was not efficiently retained by the endcapped sorbent without aptamer, hence also confirming that the aptamer was required for the binding of α-syn. In contrast, when the TE RBCs lysates were analyzed with the blank sorbent, only ubiquitin, in a similar amount to that observed by AA-SPE-CE-MS, was detected. To deplete ubiquitin from the TE RBCs lysate before the analysis by AA-SPE-CE-MS, the sample was incubated off-line with blank sorbent. However, no significant differences were observed. Results were also unsatisfactory in separate experiments, when a great concentration of off-target sequence single-stranded DNA (i.e. 200 nM) was added to the TE RBCs lysate for masking. Non-specific adsorption of ubiquitin on the endcapped solid support was probably promoted by its high abundance in the TE RBCs lysate[41], but a certain affinity to the α-syn aptamer could not be discarded. Once ruled out the possibility of reducing the amount of ubiquitin in the TE RBCs lysate, a 50 µm i.d. separation capillary was tested to improve the separation resolution between α-syn and ubiquitin. However, loading the TE RBCs lysate for AA-SPE-CE-MS promoted capillary blockage and current breakdowns during the separations, due to the complexity of the sample matrix and capillary narrowness. Therefore, the 75 µm i.d. separation capillary was necessary to analyze the TE RBCs. Under the optimized conditions, consecutive analyses of the TE RBCs lysate by AA-SPE-CE-MS were repeatable in terms of migration times and peak areas (%*RSD*s (n=3) were 6.7 and 10.8%, respectively) and the lifetime of the microcartridges was halved (around 10 analyses) compared to standards due to the

higher sample matrix complexity. The estimated concentration of the endogenous *N*-acetylated α-syn detected in the TE RBCs lysate was 0.6 $\mu g \cdot mL^{-1}$, calculated by comparison of the AA-SPE-CE-MS analyses for non-spiked and spiked samples with free α-syn standard at 0.3 $\mu g \cdot mL^{-1}$.

The developed AA-SPE-CE-MS method was applied to the analysis of TE RBCs lysate samples from blood of healthy controls and Parkinson's disease (PD) patients with stage III or IV of the disease. In both healthy controls and PD patients, ubiquitin and *N*-acetylated α-syn were detected, but no other α-syn proteoforms, including PTMs or C-terminal truncated forms. To complement and validate the results at the intact protein level by AA-SPE-CE-MS and the limited information obtained about the most abundant proteins of the differently pretreated RBCs lysates by CE-MS without on-line AA-SPE (Table S-2), the TE RBCs lysate samples were digested with trypsin and analyzed by LC-Orbitrap-MS/MS. As can be seen in Table S-3, this bottom-up indirect approach provided more information, which resulted in the identification of 156 proteins in both groups of samples (no significant differences were found between PD patients and controls). The most relevant identified proteins in accordance to emPAI values were: hemoglobin subunit alpha, *N*-acetylated α-syn, which was the only α-syn proteoform detected, apolipoprotein A-I, hemoglobin subunit beta, and apolipoprotein A-IV. Considering these results, the most probable form of the detected ubiquitin was ubiquitin-40S ribosomal protein S27a (P62979). Furthermore, it was confirmed the great complexity of the TE RBCs lysate, as well as that *N*-acetylated α-syn was the main α-syn proteoform in RBCs. Therefore, the abundance of other minor proteoforms in RBCs from healthy controls and PD patients might be very low, in agreement with recent reports[28,31]. However, a larger set of samples must be analyzed to confirm this preliminary observation made from a small set of samples. At the moment, the

presence of a high abundance of these typically very minor proteoforms, has been only confirmed for phosphorylated α-syn at Ser-129 in LB deposited in the brain of patients with different synucleinopathies, including PD[25,26].

Concluding remarks

We have developed an on-line AA-SPE-CE-MS method for the purification, preconcentration, separation, and characterization of blood α-syn. Under the optimized conditions with standards, microcartridge lifetime (around 20 analyses/microcartridge) and repeatability (2.1 and 5.4 %*RSD* for migration times and peak areas) were good, the method was linear between 0.5 and 10 $\mu g \cdot mL^{-1}$ and LOD was 0.2 $\mu g \cdot mL^{-1}$ (100 times lower than by CE-MS, 20 $\mu g \cdot mL^{-1}$). Regarding the analysis of blood samples, an off-line thermal pretreatment was necessary to remove the most abundant proteins in RBCs lysate. The AA-SPE-CE-MS method performed also reasonably well for the analysis of RBCs lysates despite the complexity of the sample matrix. However, repeatability was slightly smaller (%*RSD*s (n=3) were 6.7 and 10.8% for migration times and peak areas, respectively), microcartridge lifetime was shorter (around 10 analyses/microcartridge), total analysis times increased 10 minutes and non-specific adsorption of mainly ubiquitin was observed. In view of the results achieved, because of the high affinity to bind to the target molecule and the improved tolerance to acidic and basic conditions, immobilized aptamers can be regarded as a powerful alternative to antibodies for SPE-CE-MS. With regard to the clinical implications of the case study, in the RBCs lysate *N*-acetylated α-syn was detected to be the main proteoform in healthy controls and stage III-IV PD patients, and no other minor proteoforms were detected. Therefore, no apparent alteration of blood α-syn proteoforms at the level of a few hundreds of $ng \cdot mL^{-1}$ seems to occur during PD. In the future, a larger set of blood samples could be analyzed

to confirm this preliminary finding. Furthermore, the developed AA-SPE-CE-MS method could be applied to the analysis of α-syn from LBs isolated from the brain of patients with different synucleinopathies to screen for characteristic α-syn proteoforms.

Supporting Information

Preparation of RBCs lysates, optimized MS parameters, quality parameters, LC-Orbitrap-MS/MS analysis, M_r of the detected proteins, AA-SPE-CE-MS for filtered RBCs lysate and for ethanol-chloroform extracted RBCs lysate, CE-MS and AA-SPE-CE-MS for TE RBCs lysate and most relevant detected proteins in TE RBCs lysates after digestion with trypsin and LC-Orbitrap-MS/MS analysis.

Acknowledgements

This study was supported by a grant from the Spanish Ministry of Economy and Competitiveness (RTI2018-097411-B-I00) and the Cathedra UB Rector Francisco Buscarons Úbeda (Forensic Chemistry and Chemical Engineering). Roger Pero-Gascon acknowledges the Spanish Ministry of Education, Culture and Sport for a FPU (Formación del Profesorado Universitario) fellowship. Maxim V. Berezovski thanks the NOVA DOMUS – CHEMEDPHO project funded by the Erasmus Mundus Action II of the European Commission and the Faculty of Chemistry of the UB for the travel grants.

We thank Dr. Miguel Angel Vesga and Dr. Javier Ruiz Martínez from the node that the Basque Biobank has in Biodonostia for providing the blood samples from healthy controls and PD patients, respectively.

The authors declare no competing financial interest.

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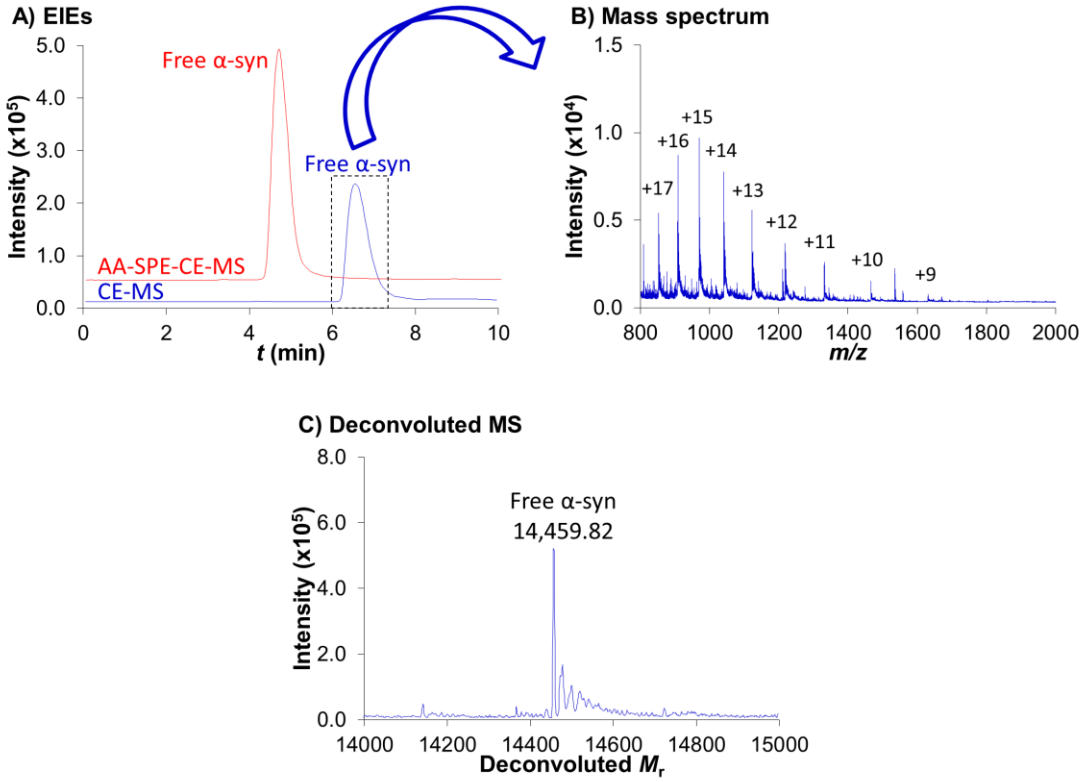


Figure 1. CE-MS and AA-SPE-CE-MS for a 100 and a 1 µg·mL^{-1} α-syn standard, respectively. **(A)** Extracted ion electropherograms (EIEs), **(B)** mass spectrum of the boxed time region (CE-MS), and **(C)** deconvoluted mass spectrum (CE-MS).

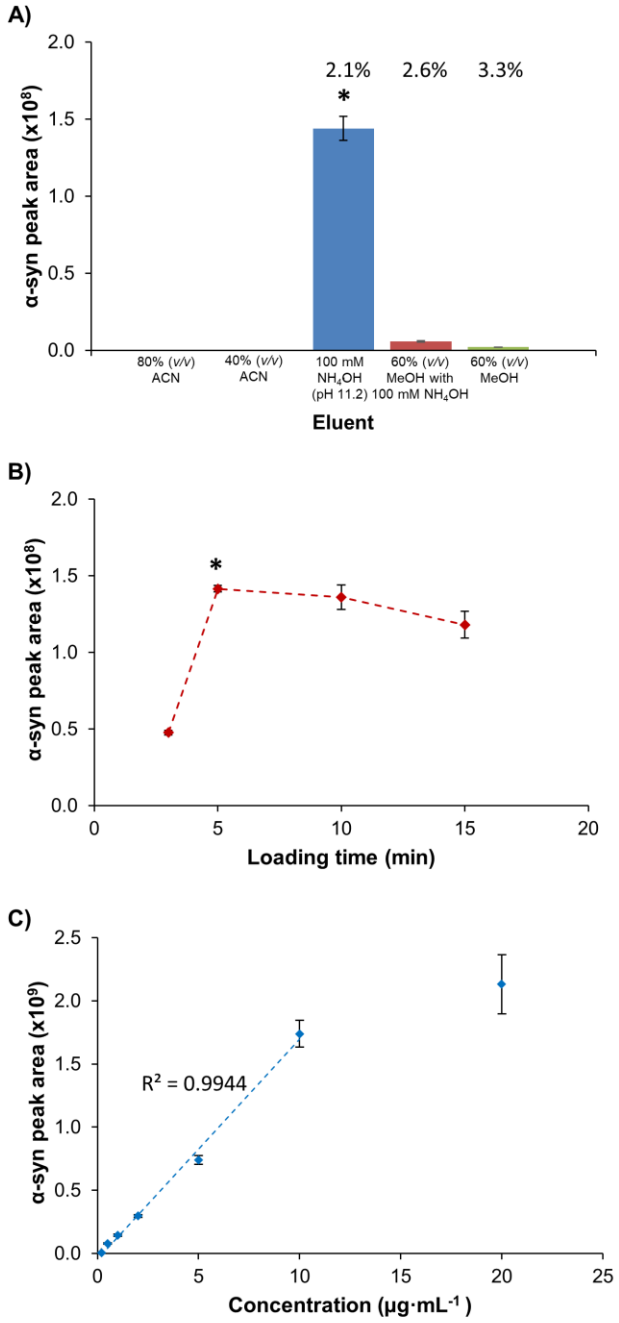


Figure 2. (A) Peak area of the detected α-syn for a 1 µg·mL^{-1} α-syn standard loaded for 5 min at 930 mbar in water by AA-SPE-CE-MS using different eluents and a BGE of 100 mM HAc (pH 2.9). **(B)** Plot of peak area of the detected α-syn vs loading time at 930 mbar (1 µg·mL^{-1} α-syn standard in water, using the optimized elution and separation conditions). **(C)** Plot of peak area of the detected α-syn vs concentration of the loaded standard solution, regression line and R^2 value using the optimized loading, elution and separation conditions. All measurements were performed in triplicate (standard deviation is given as error bars; %*RSD* for migration times is given in numbers). Optimized conditions are indicated in **(A)** and **(B)** with an asterisk.

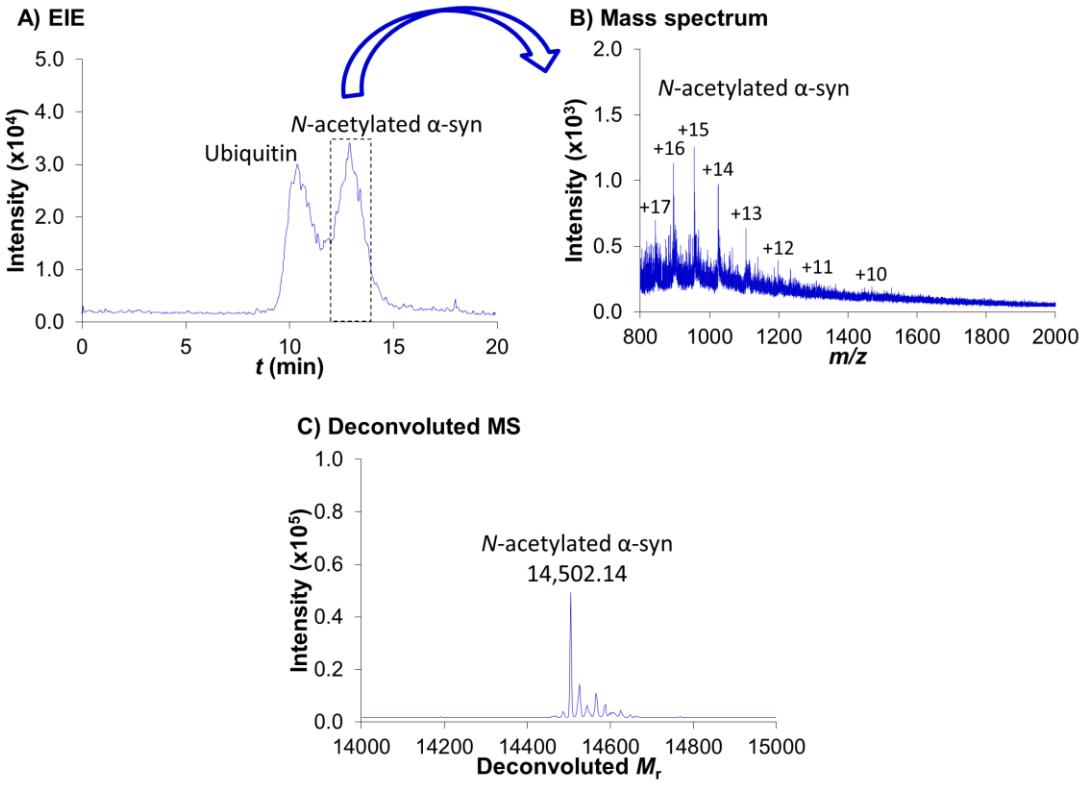


Figure 3. AA-SPE-CE-MS for a thermo-enriched red blood cells (TE RBCs) lysate sample. **(A)** Extracted ion electropherogram (EIE), **(B)** mass spectrum of the boxed time region, and **(C)** deconvoluted mass spectrum. In addition to ubiquitin, very small amounts of apolipoprotein A-I were detected (see Figure S-4).

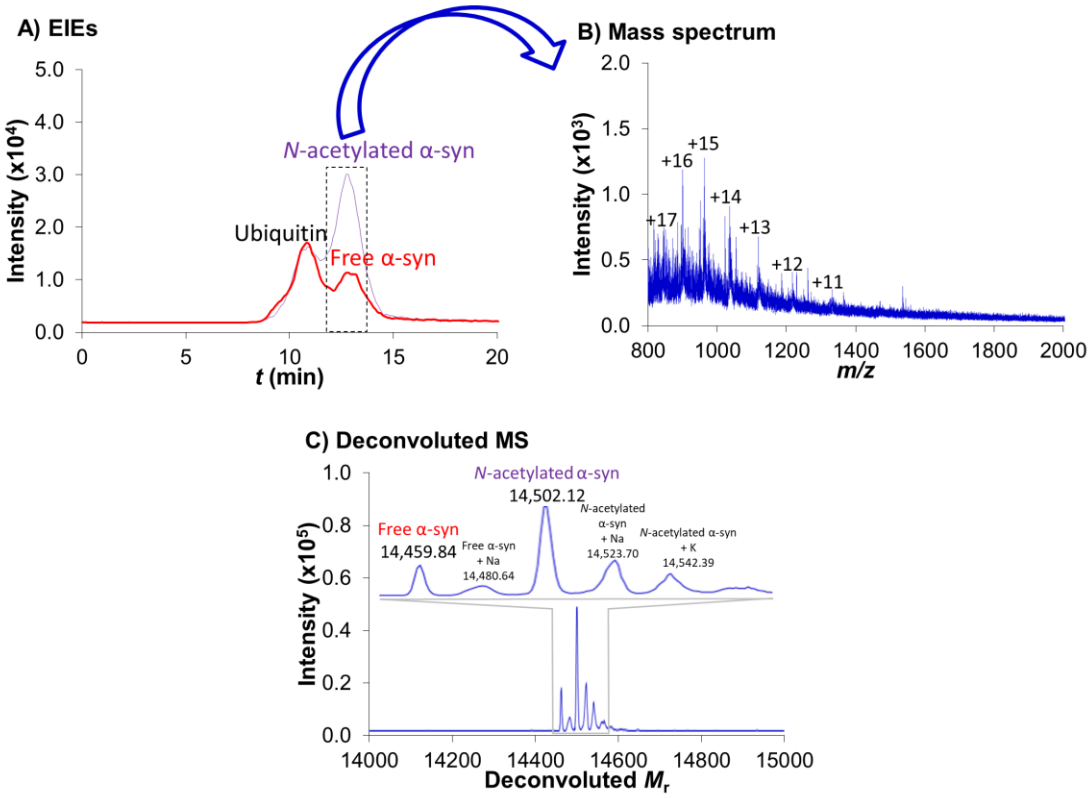
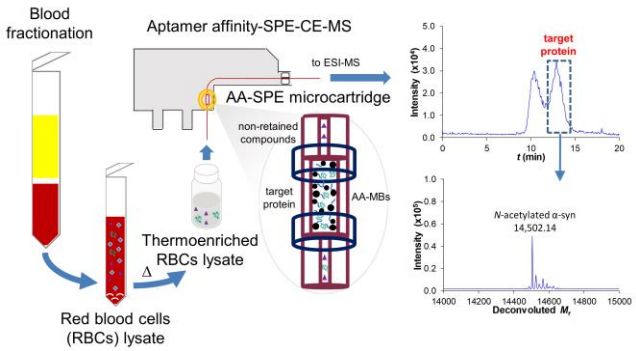


Figure 4. AA-SPE-CE-MS for a thermo-enriched red blood cells (TE RBCs) lysate sample spiked with 0.3 μg·mL^{-1} of free α-syn standard. **(A)** Extracted ion electropherograms (EIEs) for *N*-acetylated α-syn (purple) and free α-syn (red), **(B)** mass spectrum of the boxed time region, and **(C)** deconvoluted mass spectrum (see the interpretation of the zoomed M_r region).



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Supporting Information

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Preparation of red blood cells (RBCs) lysates

Fresh blood was obtained by venipuncture and was collected into 4 mL BD Vacutainer® K_2EDTA Plus blood collection tubes (Plymouth, UK) to prevent coagulation. The plasma and the buffy coat were separated from the red blood cells (RBCs) by centrifugation at 500 g for 10 min at 4ºC. RBCs were aliquoted at 250 µL in Protein LoBind 1.5 mL Tubes (Eppendorf, Hamburg, Germany), and were washed three times with 250 µL of cold isotonic 0.1 M NaCl and centrifuged at 500 g for 5 min at 4ºC. Washed RBCs from healthy controls and PD patients were separately pooled, aliquoted and stored at -80ºC until further use. To obtain the RBCs lysate, 600 µL of ice-cold water was added to 200 µL of the washed RBCs. The mixture was shaken for 10 min, and centrifuged at 6000 g for 10 min at 4ºC. The supernatant (i.e. RBCs lysate) was collected and the membranes were discarded.

Optimized MS parameters

The TOF-MS parameters were optimized analyzing by CE-MS a 100 $\mu g \cdot mL^{-1}$ α-syn standard solution: capillary voltage 4000 V, drying gas temperature 300 ºC, drying gas flow rate 4 $L \cdot min^{-1}$, nebulizer gas 7 psig, fragmentor voltage 325 V, skimmer voltage 80 V, OCT 1 RF Vpp voltage 300 V. Data were collected in profile at 1 spectrum/s between 100 and 3200 *m/z*, with the mass range set to high resolution mode (4 GHz).

Quality parameters

All quality parameters were calculated from data obtained by measuring migration time (t_m) and peak area from the extracted ion electropherogram (EIE) of α-syn proteoforms (considering the *m/z* of the most abundant molecular ions, i.e. ions with charges *+16*, *+15*, *+14*, *+13*). Repeatability was evaluated as the percent relative

standard deviation (%RSD) of t_m and peak areas. The LOD was obtained by analyzing low-concentration standard solution of α-syn (close to the LOD level, as determined from a S/N=3). LOQ was determined from a S/N=10. Linearity range was established by analyzing standard solutions of α-syn at concentrations between 0.2 and 20 $\mu g \cdot mL^{-1}$. The lifetimes of the microcartridges were investigated by repeatedly analyzing a 1 $\mu g \cdot mL^{-1}$ standard solution of α-syn and thermoenriched (TE) RBCs lysate samples. The microcartridge was discarded when the peak of α-syn in the EIE decreased more than 25% compared to the mean value of the first three analyses with the microcartridge under consideration.

LC-Orbitrap-MS/MS

One-hundred µL of TE RBCs lysate was evaporated to dryness using a Savant SPD-111 V SpeedVac concentrator (Thermo-Fisher Scientific, Waltham, MA, USA) and suspended in 100 µL of ice-cold extraction buffer (25 mM HEPES (pH 8.0), 1.5 M urea, 0.02% Triton X-100 and 5% (*v/v*) glycerol). The suspension was vortexed for 2 min and centrifuged for 30 s at 5000 g. Samples were reduced by addition of 3 mM tris(2-carboxyethyl)phosphine (TCEP) for 45 min at room temperature then alkylated with 15 mM iodoacetamide for 60 min in the dark at room temperature. Proteolytic digestion was performed by addition of 500 ng of Trypsin/Lis-C solution (Promega, Madison, Wisconsin, USA) and incubated under shaking at 500 rpm at ambient temperature overnight. The digestion was stopped by addition of formic acid (1% final concentration) and centrifuged at 15000 g for 2 minutes. The supernatant of digested proteins was desalted on disposable TopTip C-18 columns (Glygen, Columbia, MD, USA) and was evaporated to dryness.

All experiments were performed on an Orbitrap Fusion (Thermo Scientific) coupled to an Ultimate3000 nanoRLSC (Thermo Scientific). Protein digests were

reconstituted in 10 µL of 70% acetonitrile (ACN)/ 0.1% formic acid and separated on an in-house packed column (15 cm x 75 µm i.d. x 365 µm o.d. fused silica capillary, Polymicro Technologies) packed with C18 particles (Luna C18(2), 3 µm, 100 Å, Phenomenex, Torrance, California, USA) using a water/ACN/0.1% formic acid linear gradient at a flow rate of 0.30 µL/min (0-7 min, 2-2% ACN; 7-77 min, 2-38% ACN; 77-86 min, 38-98% ACN; 86-96 min, 98-98% ACN; 96-99 min, 98-2% ACN; 99-109 min, 2-2% ACN). Two µL of sample were injected.

The Orbitrap-MS/MS parameters in ESI+ were as follows: ion source temperature 250 °C, ionspray voltage 2.1 kV, top speed mode, full-scan MS spectra (*m/z* 350–2000) acquired at a resolution of 60,000. Precursor ions were filtered according to monoisotopic precursor selection, charge state (*+2* to *+7*), and dynamic exclusion (30 s with a ± 10 ppm window). The automatic gain control settings were $4*10^5$ for full FTMS scans and $1*10^4$ for MS/MS scans. Fragmentation was performed with collision-induced dissociation (CID) in the linear ion trap. Precursors were isolated using a 2 *m/z* isolation window and fragmented with a normalized collision energy of 35%.

Proteome discoverer 2.1 (Thermo Scientific) was used for protein identification. The precursor mass tolerance was set at 20 ppm and 0.6 mass tolerance for fragment ions. Search engine: SEQUEST-HT implemented in Proteome Discovery was applied for all MS raw files. Search parameters were set to allow for dynamic modification of methionine oxidation, acetyl on N-terminus, phosphorylation on serine, threonine and tyrosine, nitration of tyrosine and cysteine carbamidomethylation. The search database consisted of a nonredundant human protein sequence FASTA file from the UniProt/SwissProt database. The false discovery rate (FDR) was set to 0.05 for both peptide and protein identifications.

Six digested TE RBCs lysate samples were analyzed with two replicates of each sample. Proteins identified with “low” or “medium” protein confidence values or detected in less than 9 of the 12 analyses were not reported.

Table S-1. Theoretical and deconvoluted average M_r for the detected α-syn proteoforms in recombinant human α-syn standard (CE-MS) and TE RBCs lysate (AA-SPE-CE-MS).

Sample	**Detected α-syn proteoforms**	**Theoretical average M_r**	**Deconvoluted average M_r**	
			Experimental	**E_r[a] (ppm)**
Recombinant human α-syn standard	Free α-syn	14,460.02	14,459.82	-14
TE RBCs lysate	*N*-acetylated α-syn	14,502.06	14,502.14	6

a) Relative error (E_r) was calculated in ppm as: (M_r exp – M_r theo)/M_r theo × 10^6 (exp = experimental and theo = theoretical). M_r exp was obtained as an average of three replicates.

Table S-2. Theoretical and deconvoluted average M_r for the detected proteins in pretreated RBCs lysates.

Abundant proteins detected	Uniprot accession number	Theoretical average M_r	Deconvoluted average M_r		Detected						
					CE-MS			AA-SPE-CE-MS			Blank SPE-CE-MS
			Experimental	E_r[a] (ppm)	Filtration	Ethanol-chloroform extraction	Thermo-enrichment	Filtration	Ethanol-chloroform extraction	Thermo-enrichment	Thermo-enrichment
Hemoglobin subunit alpha	P69905	15,126.20	15,126.29	6	X			X			
Hemoglobin subunit beta	P68871	15,867.05	15,866.67	-24	X			X			
Ubiquitin	P62979[b]	8564.76[c]	8564.91	17		X	X		X	X	X
N-acetylated carbonic anhydrase 1	P00915	28,780.75	28,780.78	1		X			X		
Apolipoprotein A-I	P02647	28,078.33	28,078.13	-7			X			X	
N-acetylated α-syn	P37840	14,502.06	14,502.14	-6						X	

a) Relative error (E_r) was calculated in ppm as: (M_r exp – M_r theo)/M_r theo × 10^6 (exp = experimental and theo = theoretical). M_r exp was obtained as an average of three replicates.

b) This Uniprot accession number corresponds to ubiquitin-40S ribosomal protein S27a and was assigned taking into account the results of the LC-Orbitrap-MS/MS analysis (see Table S-3).

c) This M_r value agrees with a fragment (1-76) of P62979 protein. This fragment is also found in Uniprot accession numbers P0CG47 (polyubiquitin-B), P0CG48 (polyubiquitin-C) and P62987 (ubiquitin-60S ribosomal protein L40).

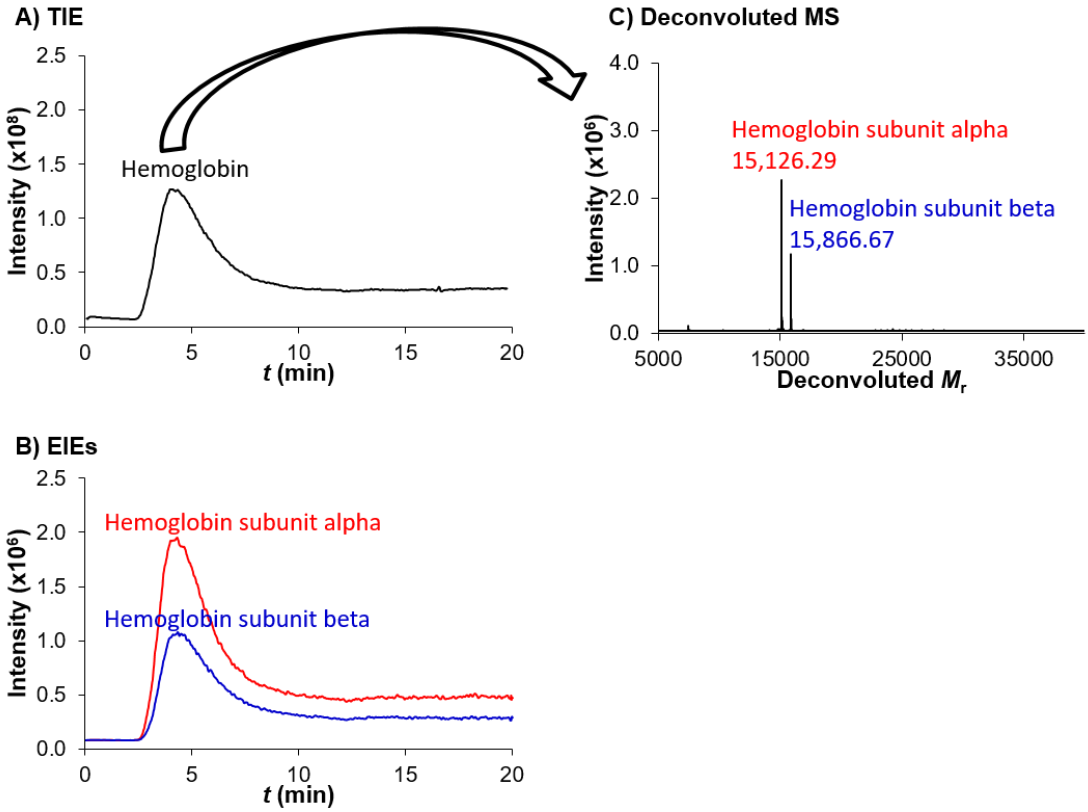

Figure S-1. AA-SPE-CE-MS for a filtered RBCs lysate. **(A)** Total ion electropherogram (TIE), **(B)** extracted ion electropherograms (EIEs), and **(C)** deconvoluted mass spectrum.

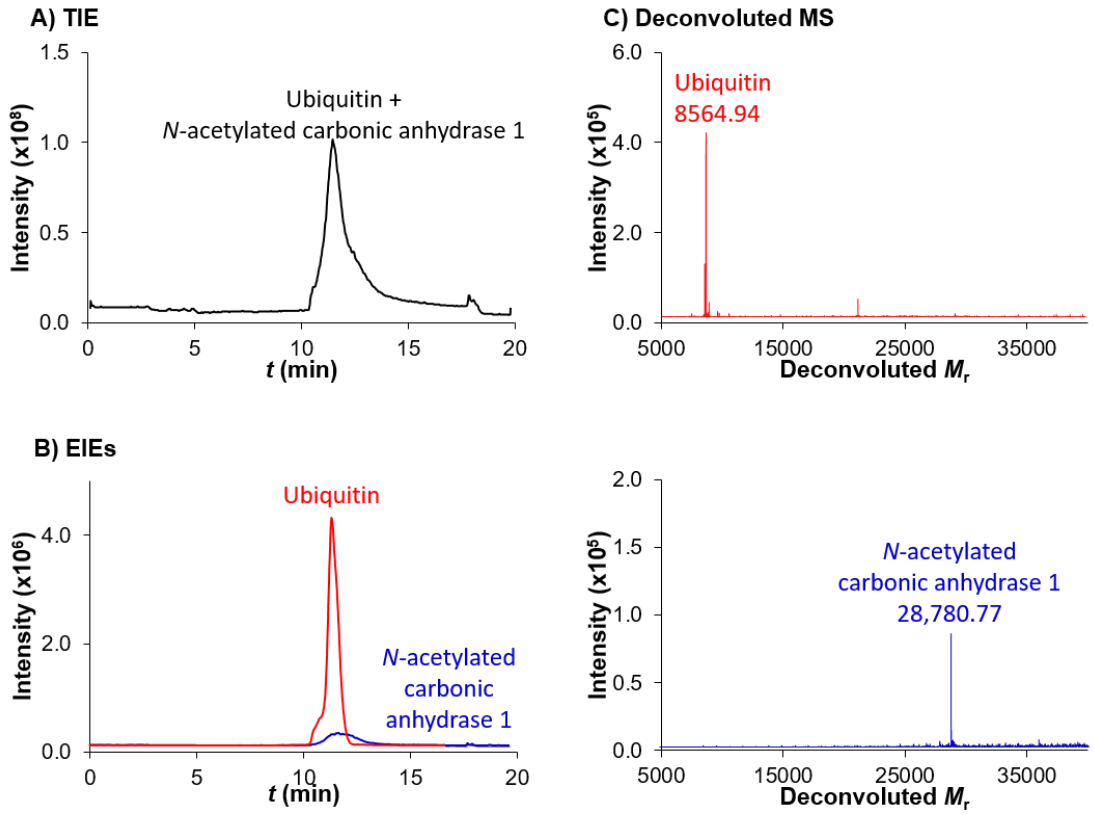

Figure S-2. AA-SPE-CE-MS for an ethanol-chloroform extracted RBCs lysate. **(A)** Total ion electropherogram (TIE), **(B)** extracted ion electropherograms (EIEs), and **(C)** deconvoluted mass spectra.

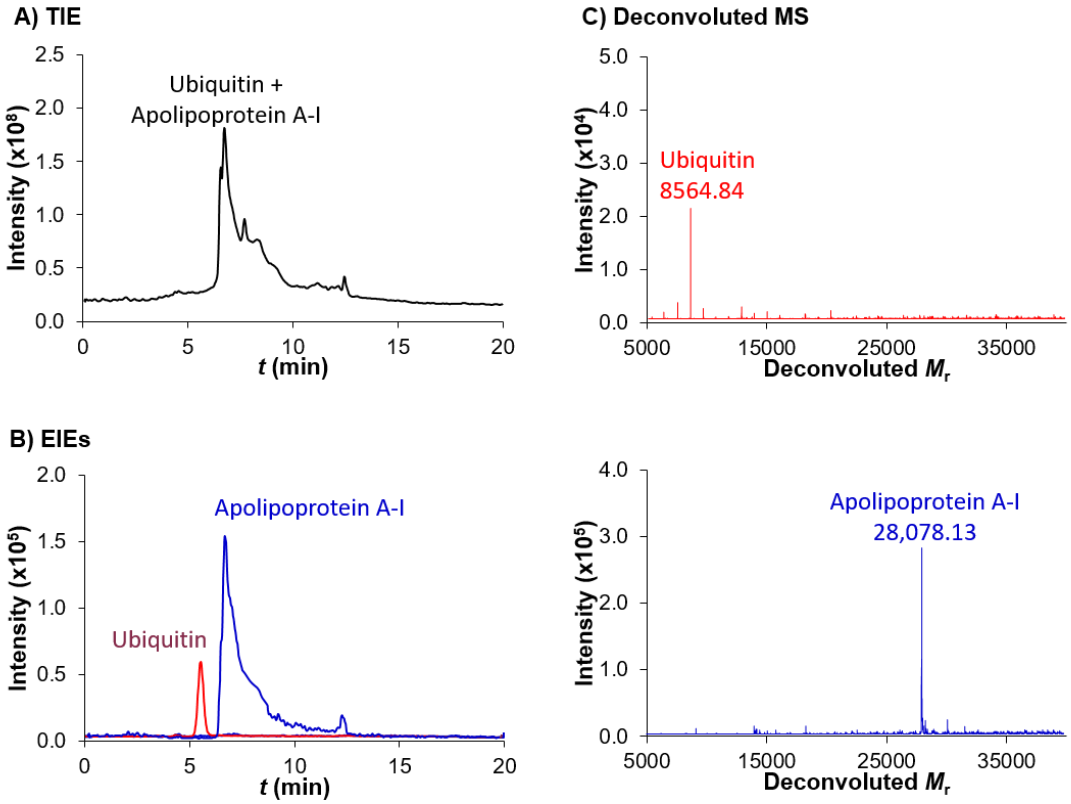

Figure S-3. CE-MS for a TE RBCs lysate. **(A)** Total ion electropherogram (TIE), **(B)** extracted ion electropherograms (EIEs), and **(C)** deconvoluted mass spectra.

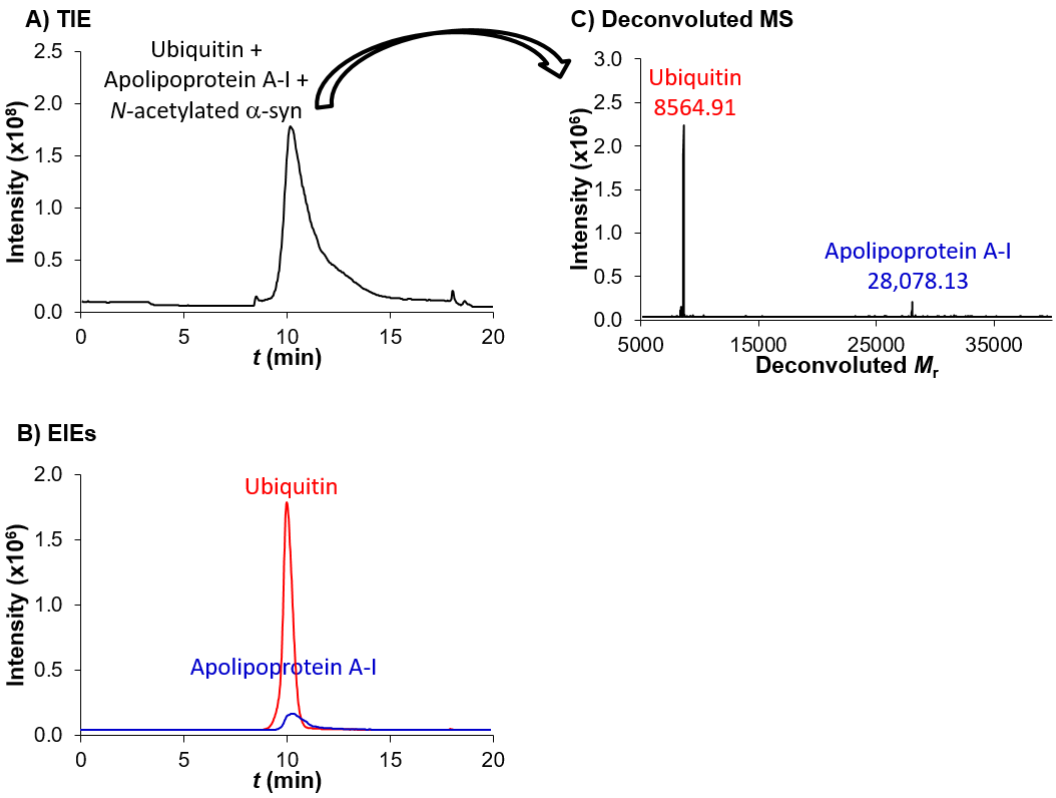

Figure S-4. AA-SPE-CE-MS for a TE RBCs lysate. **(A)** Total ion electropherogram (TIE), **(B)** extracted ion electropherograms (EIEs), and **(C)** deconvoluted mass spectrum. *N*-acetylated α-syn was also detected (see Figure 3).