

Total α -synuclein levels in human blood cells, CSF, and saliva determined by a lipid-ELISA

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Abstract The validity of α -synuclein (α -Syn) as a biomarker for Parkinson's disease (PD) is still under investigation. Conventional methods for capture and quantitation of α -Syn protein in human samples are primarily based on anti- α -Syn antibodies. Specific and competent antibodies were raised against α -Syn. However, capture by anti- α -Syn antibodies may be limited to specific epitope recognition, attributed to protein structure or post-translational modifications. Hence, antibody-based methods for α -Syn capture raise a concern regarding their efficacy to detect the intracellular, unfolded α -Syn pool. An alternative is α -Syn capture by membrane lipids, i.e., to utilize the biochemical property of α -Syn to specifically bind membrane lipids and acquire a characteristic structure following binding. We determined α -Syn levels in human samples using immobilized lipids for α -Syn capture. The lipids used for α -Syn capture consist of phosphatidyl inositol (PI), phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE). Addition of mono-sialoganglioside, GM1 ganglioside, to the immobilized lipids significantly improved α -Syn detection. Following capture, the lipid-bound α -Syn was detected using an anti- α -Syn antibody. Total α -Syn levels

in whole blood cells (WBC), cerebrospinal fluid (CSF), and saliva were determined by the lipid-ELISA method.

Keywords Parkinson's disease · α -Synuclein · Biomarkers · ELISA · Phospholipids

Introduction

Enzyme-linked immunosorbent assay (ELISA) is extensively used in scientific research and medical diagnostics. Although there are modifications to the assay, the basic immunology concept of an antigen binding to its specific antibody followed by an enzymatic reaction, which allows a quantitative detection, is preserved. ELISA assays are a powerful tool used in clinical diagnosis to detect and quantify specific biomarkers in association with diseases.

A marker for Parkinson's disease (PD), which reflects on the pathogenic mechanism, is desired. α -Synuclein (α -Syn) protein represents a potential marker for PD. α -Syn pathology, in the form of Lewy body pathology, is found in brains affected with PD [1]. In the central nervous system (CNS), α -Syn pathology is associated with the onset and progression of the disease [2, 3]. α -Syn pathology is also found in peripheral tissues [2] and suspected to precede a pathogenic spread of toxic forms of the protein to the CNS (reviewed in [4, 5]). The occurrence of α -Syn in accessible human fluids, including blood plasma [6] and blood cells [7–9], saliva [10], and cerebrospinal fluid (CSF) [11], has motivated researchers to examine the feasibility of peripheral α -Syn as a biomarker for PD [12–14].

Two independently regulated pathways of expression were reported for α -Syn: (i) neuronal-expressed, prion-like secreted α -Syn [15] and (ii) blood cells-expressed, particularly of erythroid lineage [7–9]. Importantly, growing evidence now

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supports the primary hypothesis suggested by Braak et al. [2], claiming that α -Syn pathology in peripheral autonomic neurons within the gastrointestinal system precedes CNS pathology [16–18]. Moreover, a recent study suggested that specific intravenously injected pathogenic forms of α -Syn cross the blood brain barrier into the CNS, suggesting a potential role for α -Syn expressed in blood cells in the onset of PD [19].

Attempts to quantify α -Syn protein deal with its multifaceted presentations. That is, α -Syn occurs as unstructured, natively unfolded protein in solution [20]; in various post-translational modifications [21]; and assembles in soluble oligomers and insoluble aggregates [22–24]. The debate concerning pathogenic vs. physiologic forms of α -Syn is still ongoing and therefore it is important to detect a wide range of α -Syn forms.

α -Syn normally binds membrane lipids, including negatively charged phospholipids and mono-sialoganglioside, GM1 [25–27]. α -Syn binding to membrane lipids is mediated by its N' terminus [28] and involves the KTKEGV repeat motif [29]. Notably, membrane lipid binding is necessary for the unstructured protein to obtain an α -helix-rich structure [25], which governs α -Syn's oligomerization in soluble oligomers [23, 24, 29].

We developed an ELISA-based method, which consists of α -Syn capture from a test sample by immobilized lipids, preferably phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), and GM1 ganglioside. α -Syn capture is followed by detection of the bound α -Syn, using an anti- α -Syn antibody and a quantitative enzymatic reaction of horseradish peroxidase (HRP) or electrochemiluminescence (ECL)-based detection. Total α -Syn levels are determined in whole blood cells, CSF and saliva. Assay specificity and sensitivity are described.

Methods

Lipid-ELISA

A 96-well PolySorp ELISA plate (Thermo Scientific, Getter, Israel) was coated with PI, PS, PE (SIGMA, Rehovot, Israel) and GM1 ganglioside (AVANTI, D-Chem, Petach-Tikva, Israel), dissolved in methanol at a final amount of 5 μ g/well and incubated overnight at 4 °C for complete evaporation of methanol (immobilization of lipids) (Fig. 1). Blocking was performed with 100 μ l/well of 1 % BSA (fatty acid-free) in PBS for 1 h at 37 °C, followed by one wash with PBS. Test samples were added onto the plates, in triplicates and incubated for 3 h at 37 °C to allow capture of proteins by the immobilized lipids. The wells were washed with 3 % H₂O₂

in doubly distilled water for 10 min and then with PBS containing 0.1 % CHAPS (Sigma, Rehovot, Israel). An anti- α -Syn antibody (C20, Santa Cruz Biotechnology, Almog, Israel) diluted 1:1000 in 1 % BSA (FA-free) in PBS was added to the wells. Following incubation for 1 h at 37 °C, the wells were washed three times and incubated for 1 h at 37 °C with a horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody (Jackson Laboratories, ENCO, Israel) diluted 1:8000 in 1 % BSA in PBS. An enzymatic reaction was then performed. For α -Syn detection in WBC, 50 μ l of TMB one component microwell substrate (Southern Biotech, Birmingham, Alabama, USA) was added per well. The reaction was terminated with 50 μ l/well of 1 M H₂SO₄. Absorbance at 450 nm was determined using a plate reader (EL808 Ultra Microplate Reader, Bio-Tek Instruments, VT, USA). For α -Syn detection in CSF and saliva, samples were applied on white PolySorp ELISA plates (Thermo Scientific) and Super Signal ELISA Femto (Pierce, Ornat, Israel) was used for the enzymatic reaction. Luminescence was determined by luminometer (Infinite M200 Pro, NEOTEL Scientific Instrumentation Ltd.) immediately after adding the substrate.

The amount of α -Syn was determined for each plate and each immobilized lipid combination, according to the linear phase of a standard curve consisting of recombinant α -Syn, performed in parallel to the tested samples.

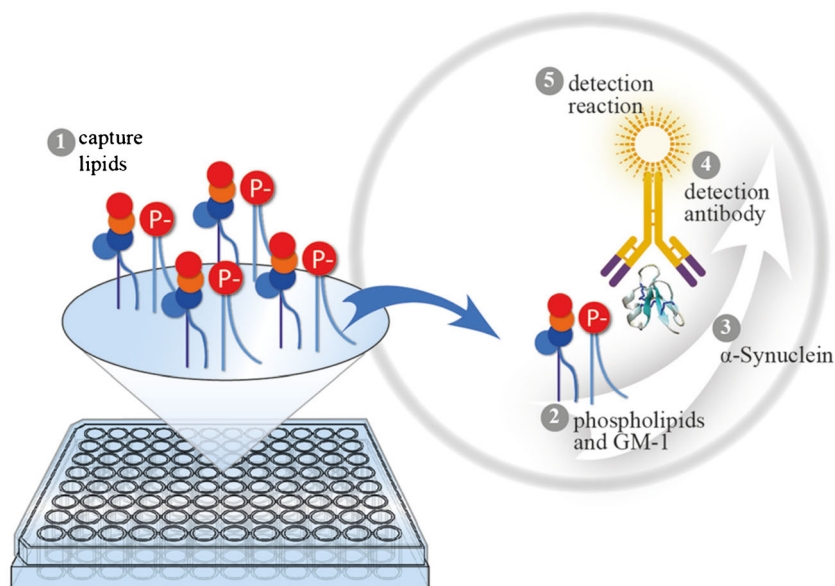
Human samples

Whole blood cell pellets (WBPs) were collected from six healthy volunteers, ages 27–51 years (three women and three men). Blood was drawn into EDTA-coated (purple top) tubes. Following spin-down at 2000 \times g for 5 min at 4 °C, plasma was removed and pelleted cells were stored at –80 °C for 24 h. The frozen pellet was then resuspended at 1:5 (v/v) in cold hypotonic buffer (10 mM Tris-HCl, pH 7.5), placed on ice for 5 min and then spun at 17,000 \times g for 30 min at 4 °C. The supernatant was collected, transferred to a clean tube and diluted 1:50 (v/v) in 1 % BSA in PBS.

CSF samples were collected by a lumbar puncture in the L3–L4 space, using a 22G needle, after overnight fasting and without medications. During extraction, CSF visually contaminated with blood was rejected. CSF was immediately centrifuged at 4000 \times g for 10 min at 4 °C and subsequently stored at –80 °C in aliquots until analyses.

Saliva samples were collected from four healthy volunteers, ages 25–40 years, at midday hours, after a 2-h fast. Samples were collected by direct expectoration (spitting) into a sterile tube. Samples were then diluted 1:1 in PBS containing 0.5 mM MgCl₂, 0.9 mM CaCl₂, 50 mM Tris-Cl pH 8.5, 0.1 % CHAPS, 1 mg/ml DNase

Fig. 1 Illustration of lipid-ELISA method. Immobilized lipids attached to the bottom of a 96-well microtiter plate are used to capture α -Syn from a test sample. The detection of captured α -Syn is performed with an anti- α -Syn ab, followed by an enzymatic reaction



and 1 mM PMSF, and incubated overnight at 4 °C with gentle shaking. Fresh samples were used for α -Syn determinations.

Immunodepletion

Samples of lysed WBCs (100 μ g protein) in 1 ml homogenization buffer [20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.32 M sucrose, 43 mM β -mercaptoethanol and a protease inhibitor mix (Sigma)]. No detergents were added to the buffer, to avoid damage to the immobilized lipids in the lipid-ELISA assay. Samples in a final volume of 1 ml were incubated with anti- α -Syn ab, syn-1 (1–2 μ g; Transduction Laboratories) for 1 h at 4 °C. Then, 40 μ l of protein A/G PLUS-Agarose (Santa Cruz, Almg, Israel) beads were added and the tube was placed on a rocker platform for 1 h at 4 °C. The tube was spun at 1000 \times g and pelleted beads were washed in PBS. Elution of α -Syn from protein A/G PLUS-Agarose was performed in acidic buffer containing 0.2 M glycine at pH 2.3 for 10 min, and neutralized with an equal volume of 1 M Tris pH 8.0. The immune-depleted sample; an identical sample, handled and treated in parallel without Syn-1 ab; and the acidic eluted sample were tested by lipid-ELISA using C20 ab for detection.

Statistical analysis

The results are presented as means \pm SDs. *P* values for differences in α -Syn detection between PL and PL/GM1 immobilized lipids were analyzed by one-way ANOVA.

Results

GM1 ganglioside improved α -Syn capture by phospholipids

A combination of immobilized phospholipids (PL), consisting of the acidic phospholipids, phosphatidyl inositol (PI) and phosphatidylserine (PS) and the zwitterionic phosphatidyl ethanolamine (PE) at 1:1:1 w/w ratio and a final amount of 5 μ g per well, was found in a previous study to effectively detect α -Syn in test samples [29]. We now tested the effect of GM1 on α -Syn detection. To this aim, a standard curve consisting of recombinant α -Syn (at 5–25 ng) was applied into a 96-well plate, precoated either with PI/PS/PE (1:1:1 w/w); with this PL mixture containing an equivalent amount of GM1 at 1 \times or 2 \times ; or with GM1 only. Control wells were treated in parallel with methanol only, the solvent used for phospholipids and GM1. Following capture, α -Syn was detected using an anti- α -Syn antibody, C20. A linear curve, presenting increasing values of optical density (OD) at 450 nm with increasing recombinant α -Syn concentrations, was obtained for the different immobilized lipids. Maximal α -Syn detection was obtained with PI/PS/PE/GM1 (w/w) followed by PI/PS/PE. α -Syn detection by the other tested combinations of PL and GM1 appeared less efficient (Fig. 2a). Detection of α -Syn was next compared at lower concentrations (25–200 pg) measuring luminescence. A linear curve representing increases in luminescence as a function of recombinant α -Syn levels was obtained using PI/PS/PE/GM1 ($R^2 = 0.098$) and PI/PS/PE ($R^2 = 0.095$) (Fig. 2b).

To set the limit of α -Syn detection by the lipid-ELISA method, we determined the minimal α -Syn amount, which is above the assay's baseline. To this aim, the baseline was

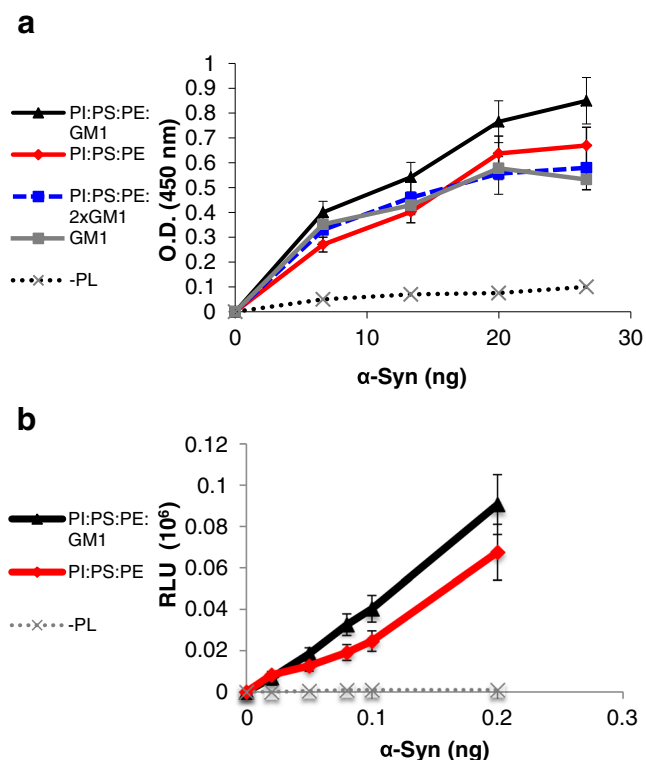


Fig. 2 Detection of human recombinant α -Syn by the lipid-ELISA assay. **a** Human recombinant α -Syn (5–25 ng) was applied in microtiter wells, precoated with the specified lipids (at a final amount of 5 μ g/well) or in control wells, without lipids. Following capture by lipids, bound α -Syn was detected by anti- α -Syn ab, C20 (Santa Cruz Biotechnology) and quantified at 450 nm (see “Methods”). **b** Human recombinant α -Syn (25–200 pg) captured using the specified lipids. Bound α -Syn determined by C20 ab and an enzymatic reaction coupled to luminescence and presented in relative luminescence units (RLU). Mean of three replicates \pm SD. *PI* Phosphatidyl inositol, *PS* phosphatidyl serine, *PE* phosphatidyl ethanolamine, *GM1* monosialoganglioside

determined in eight wells and subtracted from the values obtained for increasing amounts (in picograms per milliliter) of purified recombinant α -Syn protein. The minimal detectable α -Syn level was determined as the amount of α -Syn that corresponded to a value that was at least two standard deviations above the baseline [30]. The minimal α -Syn amounts detectable by the lipid-ELISA (in picograms of α -Syn per milliliter) are shown in Table 1. The data represent four different experiments performed on different days.

These numbers indicated an approximately 2- to 3.2-fold higher sensitivity for α -Syn detection using PLs

Table 1 Limit of α -Syn detection by lipid-ELISA

	Absorbance at 450 nm ^a	Luminescence ^a
PI/PS/PE/GM1	8.1 \pm 1.9	3.7 \pm 0.2
PI/PS/PE	26.0 \pm 2.1	7.6 \pm 0.2

^a Recombinant α -Syn (pg/ml)

Table 2 Total α -Syn levels detected in human samples by the lipid-ELISA

α -Syn levels ^a	PI/PS/PE	PI/PS/PE/GM1	Sample dilutions
WBC (450 nm)	25,266.5 \pm 5445	32,031.2 \pm 6248	1:500
CSF (luminescence)	11.2 \pm 4.1	16.7 \pm 5.9	–
Saliva (luminescence)	13.5 \pm 3.5	55.9 \pm 10.3	1:50

^a α -Syn amounts in ng/ml

plus GM1 than PLs only. The use of luminescence further increased assay sensitivity in both lipid combinations.

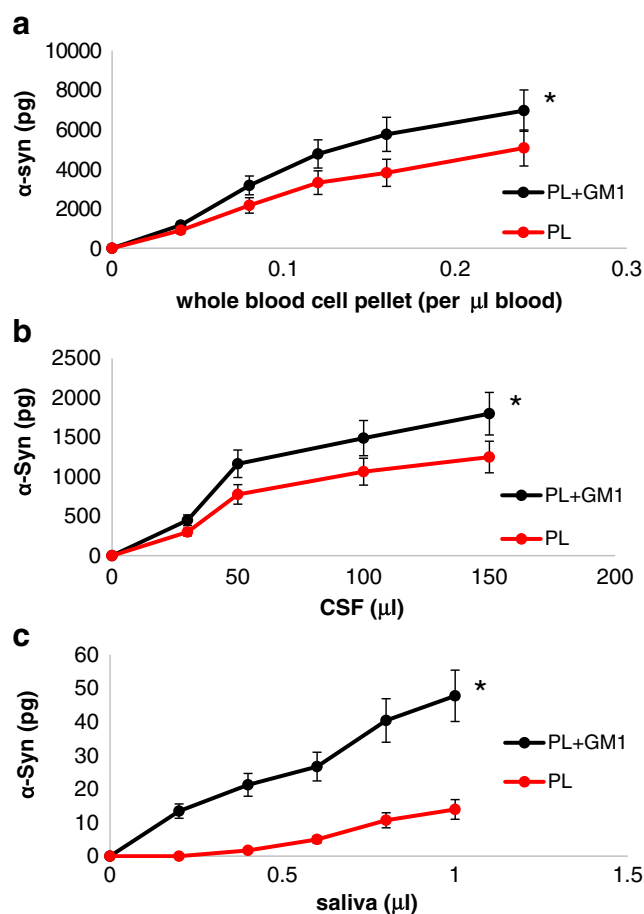


Fig. 3 α -Syn detection in human samples. Samples of whole blood cell pellet were applied in amounts corresponding to 0–0.25 μ l of the original blood sample. α -Syn levels were detected using the lipid-ELISA and quantified at 450 nm (**a**); CSF samples were directly applied at 0–150 μ l. α -Syn levels quantified by luminescence (**b**); saliva samples applied in amounts corresponding to 0.2–1 μ l of the original saliva. α -Syn levels quantified by luminescence (**c**). α -Syn levels were calculated according to a standard curve, consisting of recombinant α -Syn and performed in parallel to the test sample, in the same plate. *PL* PI/PS/PE (*PI* phosphatidyl inositol, *PS* phosphatidyl serine, *PE* phosphatidyl ethanolamine); *PL* + *GM*, PI/PS/PE/GM1 (monosialoganglioside). Mean \pm SD of n = 6 for WBC; n = 6 for CSF, and n = 4 for saliva. * P < 0.05, ANOVA

GM1 ganglioside in the immobilized phospholipids mix improved α -Syn detection in human samples

α -Syn levels were next determined in human whole blood cells (WBC; $n=6$), CSF ($n=6$), and saliva ($n=4$), using either PLs only or PLs/GM1 for immobilized lipids (Table 2). The calculated α -Syn amounts (in nanograms per milliliter of sample) detected by immobilized PLs (PI/PS/PE) were $25,266.5 \pm 5445.3$ in WBC, 11.2 ± 4.1 in CSF, and 13.5 ± 3.5 in saliva. Importantly, significantly higher amounts of α -Syn (in nanograms per milliliter) were detected with immobilized PLs/GM1 (PI/PS/PE/GM1). That is, $32,031.2 \pm 6248.0$ in WBC, 16.7 ± 5.9 in CSF, and 55.9 ± 10.3 in saliva. Together, the addition of GM1 to the PLs mixture improved α -Syn detection in human samples by 20–60 % (see Table 1 and Fig. 3a–c). This effect on α -Syn detection may result from an effective α -Syn binding to the capturing lipid mix or effective acquisition of a structure, allowing recognition by the anti- α -Syn antibody.

Assay linearity, specificity and matrix interference

Assay linearity was determined using samples of WBC. These samples were chosen to demonstrate assay linearity due to the enrichment of blood cells with peroxidases as well as additional proteins that may interfere with the assay. Samples of WBC were tested at 1:50, 1:100, and 1:200 dilutions using either PI/PS/PE (Fig. 4a) or PI/PS/PE/GM1 (Fig. 4b) for immobilized lipids. The results demonstrate assay linearity as a function of sample dilutions.

Specificity of α -Syn detection by the lipid-ELISA was determined by immunodepletion of α -Syn from samples of WBC, which express the highest α -Syn levels among the samples tested in this study. Effective immunodepletion (>93 %) of α -Syn was obtained with Syn-1 antibody and resulted in a dramatic reduction in α -Syn levels detectable by lipid-ELISA with C20 antibody. Similar results were obtained following immunodepletion of α -Syn with C20 antibody and detection in lipid-ELISA with Syn-1 antibody (Fig. 4c, d). This result therefore suggests that the signal obtained by lipid-ELISA is α -Syn specific.

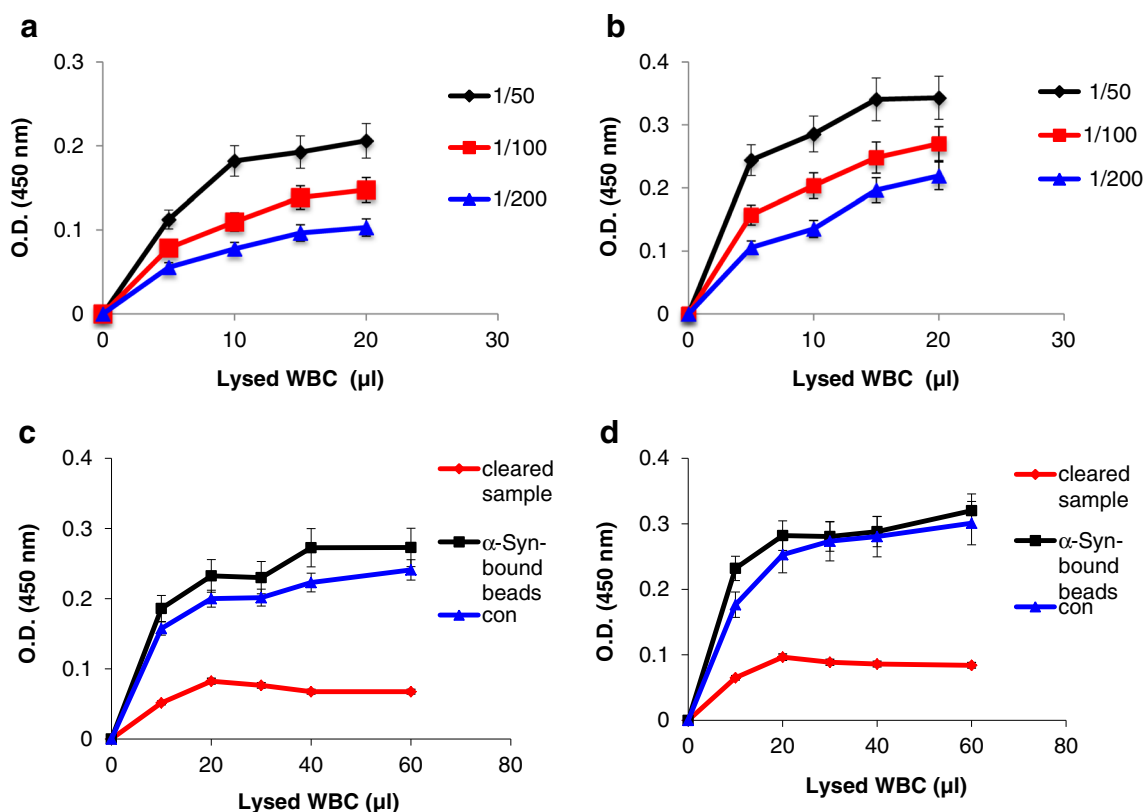


Fig. 4 Assay specificity and linearity in whole blood cells. Samples of WBC were analyzed at increasing dilutions (1:50, 1:100 and 1:200) and α -Syn levels were determined with PI/PS/PE (a) or PI/PS/PE/GM1 (b). Mean \pm SD of three replicates. A sample of WBC (100 μ g protein) was immunodepleted with the anti- α -Syn ab Syn-1 (1–2 μ g, Transduction Laboratories). Following immunodepletion, α -Syn was eluted from the

A/G Agarose beads with glycine (0.2 M) at pH 2.3 and neutralized with an equal volume of Tris-Cl pH 8.0 (1.0 M). The cleared sample and eluted beads were analyzed in parallel to a non-treated WBC sample (con) using immobilized PI/PS/PE (c) or PI/PS/PE/GM1 (d). A representative graph of three experiments is shown; mean \pm SD of three replicates

A strong matrix interference with α -Syn detection was initially observed in saliva samples. To remove this inhibition, we pretreated saliva samples to dissolve salivary mucus (see “Methods”) and examined the effect of sample dilutions. Recombinant α -Syn (500 pg) was spiked into pretreated saliva samples diluted 1:10, 1:50 or 1:100, and detectable α -Syn levels were compared with 500 pg recombinant α -Syn in PBS, determined using immobilized PL/GM1 (Fig. 5a). The results show that α -Syn detection was inhibited in saliva samples diluted 1:10 or 1:50, but no signs of interference were observed at 1:100 dilution. That is, the signal obtained for 500 pg of recombinant α -Syn in PBS was lower than the signal obtained for 500 pg spiked into a sample of 1:100 saliva, suggesting the detection of endogenous salivary α -Syn in addition to the spiked-in recombinant protein. Similar results were obtained with spiking 200–1000 pg α -Syn into 1:100 diluted samples (not shown).

Matrix interference was also tested in WBC samples. Recombinant α -Syn was spiked at 100–5000 pg into 50 μ l

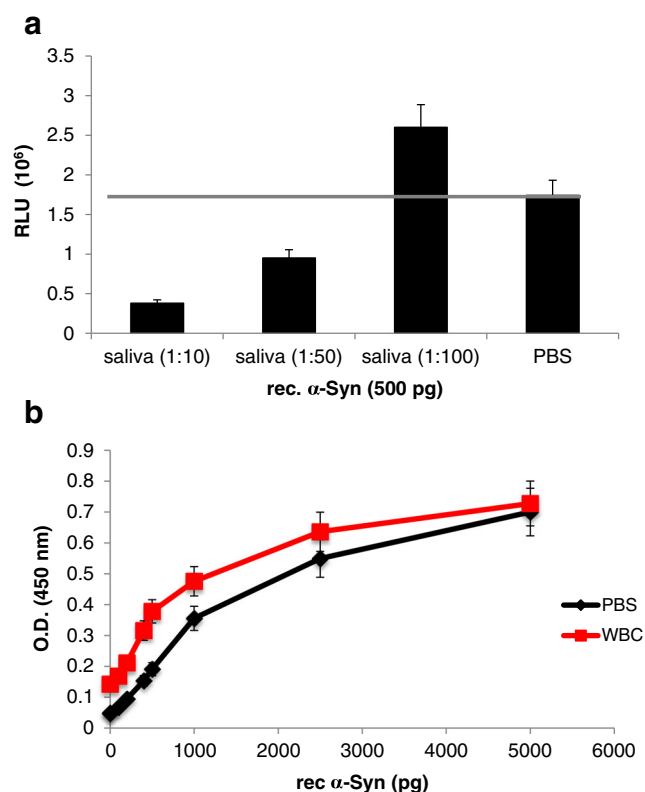


Fig. 5 Removal of matrix interference. (a) Recombinant α -Syn (500 pg) was spiked into samples of saliva, diluted 1:10; 1:50 or 1:100. α -Syn levels were determined with immobilized PI/PS/PE/GM1 with reference to 500 pg recombinant protein in PBS (horizontal line). Apparent matrix inhibition presented in saliva samples at 1:10 and 1:50 dilutions but not in 1:100 dilution. Mean \pm SD of relative luminescence units (RLU) of three replicates. (b) Curves exemplifying recombinant α -Syn at 100–5000 pg in PBS or spiked into 50 μ l of 1:1000 diluted WBC sample. The endogenous α -Syn in the WBC sample is represented by the gap between the curves at the linear phase

of WBC sample, pretreated to remove peroxidases, and diluted 1:1000. The curve obtained for recombinant α -Syn in BSA/PBS paralleled the curve in the WBC sample up to the highest point of 5000 pg, where the two curves reached a plateau (Fig. 5b). The higher signal obtained with no added recombinant α -Syn and throughout the linear phase of the curve (up to 1000 pg) represents the endogenous α -Syn in the WBC sample. Together, the results suggest that there is no matrix interference in samples of WBC.

Assay linearity and immobilized lipids

The detection of α -Syn in samples of WBC was tested with increasing amounts of immobilized lipids at: 0, 2.5, 5, 7.5, and 10 μ g/well, either with PI/PS/PE (1:1:1 w/w; Fig. 6a) or PI/PS/PE/GM1 (1:1:1:1 w/w; Fig. 6b). Samples of WBCs ($n = 4$) were tested in a constant amount of 50 μ l, diluted 1:500 or 1:1000. The data presented in Figs. 6a and b show an increase in α -Syn detection, exemplified by OD at 450 nm, with increasing amounts of lipids up to 2.5 μ g per well for the PL mixture and up to 5 μ g per well for the PL + GM1 mixture. α -Syn detection then reached a plateau and further increase in total lipid amounts did not result in higher α -Syn signals. Importantly, the effect of sample dilutions is clearly shown both at the linear and plateau range of the graph. We therefore set the amount of immobilized lipids at 5 μ g/well.

The effect of antibody dilutions on α -Syn detection was tested using C20, anti- α -Syn ab, diluted at 1:500 and 1:1000. Samples of WBC ($n = 4$) at a constant amount (50 μ l of 1:500 dilution) were tested with increasing amounts of immobilized lipids (as above). The data presented in Fig. 6c and d show very similar levels of α -Syn detection using both antibody dilutions. The higher antibody dilution (1:1000) was chosen for further experiments.

Discussion

We determined total α -Syn levels in human biological fluids utilizing immobilized lipids, including phospholipids and GM1 ganglioside, for protein capture. The efficacy of α -Syn detection was compared between phospholipids only (PI, PS and PE) or these phospholipids together with GM1 ganglioside. The results showed higher α -Syn levels when GM1 ganglioside was added to the PLs. Total α -Syn levels, detected by C20, polyclonal anti- α -Syn ab, were determined in whole blood cell pellets, CSF, and saliva. These body fluids differ significantly in their amount of α -Syn and the degree of interference with α -Syn detection. To deal with these differences, a functional range of linearity was determined for each of these sources.

α -Syn is a favorable candidate biomarker for early and accurate diagnosis of PD, to measure disease progression

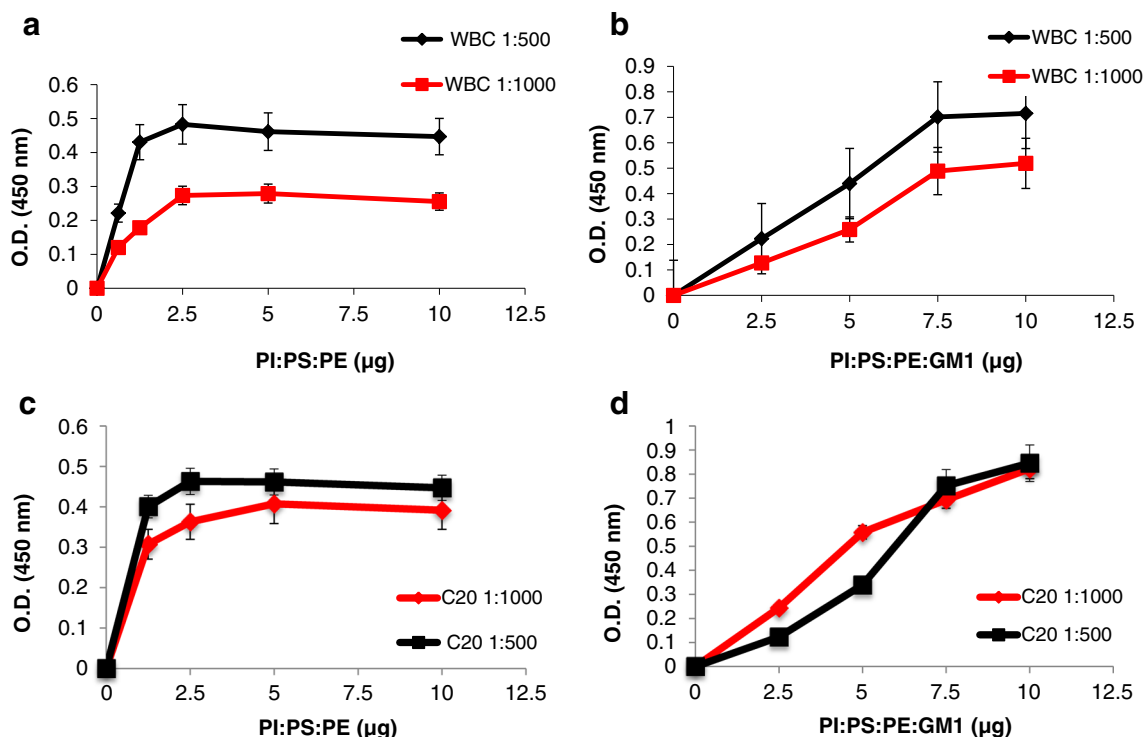


Fig. 6 Assay development. The wells of a microtiter plate were coated with 0, 2.5, 5, 7.5, or 10 μ g/well of PI/PS/PE (a) or PI/PS/PE/GM1 (b). WBC samples were applied at 1:500 or 1:1000 dilutions and α -Syn detection was performed by lipid-ELISA. Mean \pm SD of three

replicates. Samples of WBC (diluted 1:500) were applied in wells coated with PI/PS/PE (c) or PI/PS/PE/GM1 (d) at the indicated amounts. α -Syn detection was performed with two dilutions of anti- α -Syn ab, C20 (Santa Cruz Biotechnology), 1:500 and 1:1000

and response to therapy. Nevertheless, attempts to examine the value of total α -Syn as a biomarker for differential diagnosis reveal a high degree of variability in measurable α -Syn levels within the disease-diagnosed and control groups, resulting in a substantial overlap between the groups, which challenges its effectiveness as a diagnostic biomarker [31]. An alternative for total α -Syn measurements determines the level of specific α -Syn forms, e.g., phosphorylated [12] or oligomeric forms [32], but this approach faces similar challenges.

The lipid-ELISA is a sensitive method for α -Syn detection. In a previous study, we demonstrated a higher sensitivity for α -Syn detection using the phospholipid ELISA (consisting of PI/PS/PE for immobilized lipids) than a sandwich ELISA, in blood samples [14]. Of note, in CSF samples, we detected 16.7 ± 5.9 ng/ml of total α -Syn with PI/PS/PE/GM1 for capture. This amount is higher than that reported for α -Syn in CSF, detected by sandwich ELISA and set at 1.2 ± 0.4 ng/ml [13]. To the best of our knowledge, the values for total α -Syn in blood cells and saliva are yet to be determined by sensitive sandwich ELISAs adapted for maximal α -Syn detection. This high sensitivity is advantageous for α -Syn measurements in saliva and CSF, which harbor only low levels of α -Syn. However, red blood cells contain remarkably high α -Syn levels. The high sensitivity of the lipid-ELISA assay may provide a powerful tool to determine the levels of specific, non-abundant α -Syn forms in disease-diagnosed and control

groups, e.g., specific post-translationally modified α -Syn forms, which occur in lower levels. In a previous study, we determined total and proteinase K-resistant α -Syn levels in blood samples from patients with PD and healthy controls and showed a higher ratio of proteinase K-resistant α -Syn to total α -Syn in PD patients [29]. The detection of proteinase K-resistant α -Syn by lipid-ELISA may therefore highlight the power of this assay.

α -Syn is mostly cytosolic, while a fraction of it is detected bound to membranes [33, 34]. Studies in human brains affected with PD and in various animal and cellular PD models attempted to determine the cellular partitioning between membrane-associated and cytosolic α -Syn [35, 36]. However, these studies mostly rely on fractionation protocols and therefore demonstrate *in vitro* results. Nevertheless, similar results were recently reported by a study that estimated the *in vivo* partitioning of α -Syn in cortical neurons between the soma and vesicle-bound using an *in vivo* multi-photon imaging paradigm [34]. It is important to define the lipid composition that maximizes α -Syn capture both *in vivo* and *in vitro*. Here we show that adding GM1 ganglioside to phospholipids improves α -Syn detection. That is, α -Syn detection is approximately 20–60 % higher with GM1 ganglioside added to PI/PS/PE. It is interesting to test the effect of additional lipid classes, including plasmalogens and other sphingolipids. In addition, it is interesting to examine the potential effect of

the content of fatty acyl side chains of phospholipids and GM1 ganglioside on α -Syn capture.

The ability to differentiate pathogenic and physiologic species of α -Syn, on the basis of their lipid-binding specificities, is desired. Studies have shown that different familial PD-associated α -Syn mutations have different effects on membrane lipid binding. Specifically, lower affinity for acidic phospholipid was demonstrated for A30P. In contrast, A53T mutation only slightly lowered α -Syn affinity; E46K mutation enhanced α -Syn affinity; and H50Q had no detectable effect [28, 37]. Membrane lipid binding plays a critical role in α -Syn pathophysiology as it stabilizes an α -helix structure for the unfolded protein [25]. Moreover, it was shown that membrane lipid binding plays a role in α -Syn oligomerization in soluble oligomers, aggregation and toxicity [29, 37, 38]. Different protocols were used to determine the affinities of wild-type and α -Syn mutations to various combinations of lipids. However, current knowledge does not support or deny the possibility of a lipid composition that differentiates physiologic and pathogenic α -Syn species.

Membrane binding plays a role in α -Syn pathogenicity. The effective local concentration and the structural changes in α -Syn affect its oligomerization and aggregation, but with conflicting results. It appears that membrane composition and relative concentration of protein vs. lipid are key factors to determine the contribution of membranes to α -Syn pathogenicity [28]. Interestingly, membrane binding of PD-associated α -Syn mutations was shown to be mediated by a shorter helix, consisting of the 25 N'-terminal residues. This is in contrast to the 100 N'-terminal residues that mediate wild-type α -Syn associations with membranes [39]. We recently showed that mutations that deny α -Syn's associations with membranes have a lower degree of soluble oligomers and larger inclusions. That is, K to E substitutions at the first and second KTKEGV repeats support a role for membrane binding in the pathogenesis of α -Syn.

The associations of α -Syn with cell membranes may play a role in membrane and synaptic vesicle trafficking [40–45]. Nevertheless, the exact mechanism of action is not clear. Our group suggested that α -Syn specifically regulates clathrin-mediated endocytosis of synaptic vesicles and these findings were recently supported by those from another group [42, 43]. Other studies have suggested that α -Syn acts as a chaperone for the assembly of SNARE complex involved in exocytosis of synaptic vesicles [46]. While we now have a better understanding of the role of α -Syn in neuronal cells, the high levels of α -Syn detected in red blood cells raise questions relevant to its physiological role in the blood.

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Compliance with Ethical Standards

Conflict of interest Authors Suaad Abd-Elhadi, Misericordia Basora, Dolores Vilas, and Eduardo Tolosa have no conflict of interests.

Ronit Sharon owns patent PCT/IL2014/050191: An ELISA method for a sensitive detection of alpha synuclein, consisting of its phospholipids binding properties.

The authors declare that written consent is available for all healthy volunteers who donated blood, saliva, or CSF samples.

The authors declare that this study was approved by the appropriate ethics committee and was performed in accordance with the ethical standards.

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