ADVANCING PARKINSON'S DISEASE DETECTION WITH FLISA: A NOVEL APPROACH TARGETING MISFOLDED ALPHA-SYNUCLEIN

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ABSTRACT

A Fluorescence-Linked Immunosorbent Assay (FLISA) targeting β-cross-sheet α-synuclein protein, a crucial marker in Parkinson's Disease (PD), was assessed using *in vitro* assay with a rotenone-induced PD cellular model. The study aimed to evaluate FLISA's applicability with the inhouse developed labelled polyclonal antibody (pAb). Results are promising the competitive assay successfully distinguished misfolded α-synuclein in SH-SY5Y cells. Quantitative analysis revealed a direct correlation between competitive antigen concentration and decreased fluorescence, showcasing FLISA's sensitivity. Controls exhibited maximal fluorescence, confirming the absence of misfolded proteins, while rotenone-exposed cells displayed reduced fluorescence, suggesting their presence. This approach enhances PD understanding and supports potential interventions.

Keywords: Parkinson's disease, FLISA, misfolded α-Synuclein, early detection, neurodegenerative disorder, SH-SY5Y cells, rotenone-induced model

INTRODUCTION

In the realm of diagnostics and biomedical research, the field of fluorescence-linked immunosorbent assays (FLISAs) has emerged as a powerful and versatile tool. FLISAs offer precise, sensitive, and quantitative detection of specific molecules, making them invaluable in diverse applications ranging from clinical diagnostics to drug discovery.

Parkinson's Disease (PD), a complex neurodegenerative disorder characterized by the aggregation of misfolded alpha-Synuclein (α-Synuclein) protein, poses significant challenges for early diagnosis[1–4]. Currently, the clinical diagnosis of PD primarily relies on the observation of motor symptoms, which typically manifest only after substantial neuronal damage has occurred. This delay in diagnosis hinders the timely implementation of therapeutic interventions that could potentially slow or halt disease progression[2,5,6].

To address this pressing need for earlier and more accurate PD diagnosis, this study harnesses the capabilities of FLISAs. FLISAs are a family of highly sensitive immunoassay techniques that leverage the unique properties of fluorescence for the quantification of specific molecules. In the context of PD, FLISAs hold immense promise for detecting the misfolded β-cross-sheet conformation of α-synuclein, a pivotal hallmark of the disease[7–11].

The development of a robust and selective polyclonal antibody (pAb) against β-cross-sheet α-synuclein, coupled with innovative competitive NC-FLISA (Nitrocellulose Membrane-Based Competitive FLISA) assays, forms the core of this research endeavour[12]. These assays enable the accurate and efficient detection of β-cross-sheet α-synuclein, even in its earliest stages, potentially revolutionizing the landscape of PD diagnosis.

In this paper, we embark on a journey to explore the immense potential of FLISA-based techniques in advancing our understanding of α-synuclein misfolding, aggregation, and its role in PD pathogenesis[13,14].

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Furthermore, we aim to demonstrate the utility of the developed FLISA assays as a non-invasive and sensitive diagnostic tool for the early detection of PD. To demonstrate the practical utility of this assay, we turned our attention to an *in vitro* model of PD induced by rotenone in SH-SY5Y cells.

Rotenone, a potent mitochondrial complex I inhibitor, has been widely employed to create cellular models that mimic the pathological features of PD. In this model, SH-SY5Y cells were exposed to rotenone, leading to a cascade of events mirroring key aspects of PD pathogenesis[15].

Following a rigorous experimental protocol, we evaluated the applicability of our developed FLISA in the context of this cellular PD model. We aimed to assess whether our FLISA could effectively detect and quantify β-cross-sheet α-Synuclein in a biologically relevant system, representative of PD pathology.

The implications of this research extend beyond PD, as FLISA-based approaches have the potential to transform the field of neurodegenerative disease diagnostics and biomarker discovery, paving the way for more effective and timely interventions in these devastating conditions.

MATERIALS AND METHODS

Chemicals

In this study, α-synuclein in both β-cross-sheet and monomeric forms was procured from the Indian Institute of Technology, Mumbai, Maharashtra, India. Nitrocellulose membranes (8 x 8 cm) were obtained from Thermo Fisher Scientific, Bangalore, India. The pAb was developed in-house at the Institute of Chemical Technology, Mumbai, India, for experimental use. Additionally, VivoTag 680 XL, procured from PerkinElmer, Mumbai, India, was tagged with the developed pAb to facilitate analysis. SHSY5Y cells used in the study were acquired from the National Centre for Cell Science, Pune, India. Furthermore, all chemicals and reagents were supplied by the commercial supplier Shree Sai Enterprises, Thane, Mumbai, India. This comprehensive sourcing strategy ensured the availability of high-quality materials for a robust and reliable research investigation.

Secondary structural confirmation of α-synuclein protein by circular dichroism spectroscopy (CD)

In a quartz cell measuring 0.1 cm in length and containing 10 µM protein, 20 mM Gly-NaOH buffer at pH 7.4 was used for CD spectroscopy (Hellma, Forest Hills, NY). Using a JASCO - 1500 instrument (USA), spectra were obtained in the 200 nm – 260 nm wavelength region. Data interval of 1.0 nm, bandwidth of 1.0 nm, scanning speed of 100 nm min^{-1}, and three accumulations were the parameters used to acquire spectra. Three separate experiments were carried out for every sample that was going to be examined. Following the manufacturer's recommendations, buffer spectra were smoothed and subtracted. All measurements were made at 25 °C[16].

Competitive NC-FLISA

In this study, an NC membrane served as the solid phase for antigen capture. Small 5mm discs were extracted from the NC membrane, and 2.5 µL of 100 ng α-synuclein β-sheets was carefully spotted on each side of the membrane, allowing them to air dry. A negative control using 100 µg $µL^{-1}$ Bovine Serum Albumin (BSA) was included. Following complete drying, the membranes were situated in a 96-well plate, subjected to a 1 h incubation with Blocking Solution (BS), and underwent three successive washes with BS for 5 minutes each.

Simultaneously, in separate tubes, a 1:1 (V/V) mixture of various concentrations of α-synuclein β-sheets and the fluorophore-tagged developed antibody was incubated for 1 h. Following this incubation, the antigen - antibody mixture was added to the wells containing the NC discs and incubated for 1.5 h. Subsequently, the discs were washed thrice with buffer solution and dried. The bound antibodies were quantified using fluorescence spectroscopy with excitation at 680 nm and emission at 700 nm[10,11].

In vitro validation assay to assess the applicability of pAb

A rotenone-induced PD model was implemented in SH-SY5Y cells to assess the applicability of the pAb. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) at 37 °C in a 5% CO_2 environment. The culture medium was refreshed every 2 days. Cells at passage number 36 were seeded at density 1 x 10^6 cells at each well in a 6-well plate until 70% confluence was attained. Following a 24 h exposure to 50 µM rotenone in duplicate wells, adherent cells underwent thorough washing with ice-cold PBS, and were subsequently lysed using 1 mL ice-cold RIPA buffer. The lysis process involved gentle scraping of the cell culture plate and agitation for 20 minutes at 4 °C. Centrifugation at 13,000 x g for 20 minutes at 4 °C clarified the lysate and the supernatant containing proteins was isolated and stored at -80 °C for further analysis[15,17].

The supernatant was evaluated via competitive NC-FLISA. In the same way, 100 µg of α-synuclein β-sheets were immobilized onto the discs, and incubated with BS for 1 h. Concurrently, separate tubes held a 1:1 (V/V) mixture of cell culture supernatants (including control, rotenone - induced PD at 24 h and 48 h) and the fluorophore-tagged developed antibody and were incubated for 1 h. Subsequently, the antigen-antibody mixtures were added to the wells containing the NC discs and incubated for 1.5 h. Following incubation, the discs underwent triple washing with BS and were dried. Quantification of bound antibodies was performed using fluorescence spectroscopy with excitation at 680 nm and emission at 700 nm[15,17].

RESULTS

Secondary structural confirmation of α-synuclein protein by circular dichroism spectroscopy (CD)

CD spectroscopy is used in determining the two different forms (monomer and β-cross-sheet α-synuclein) secondary structure of proteins. α-synuclein is a natively unstructured protein, which shows a random coil structure in the solution. During aggregation of α-synuclein, it converts from random coil structures to the β-sheet structure. CD analysis of monomeric α-synuclein protein showed negative minima at ~200 nm, which is a characteristic feature of random coil structure (Fig. 1). This confirms the secondary structure of the monomeric form of the protein. As the protein starts misfolding, the first misfolded form is the β-cross-sheet secondary structure, which is evident by the presence of a single negative peak ~218 nm in CD spectra (Fig. 1).

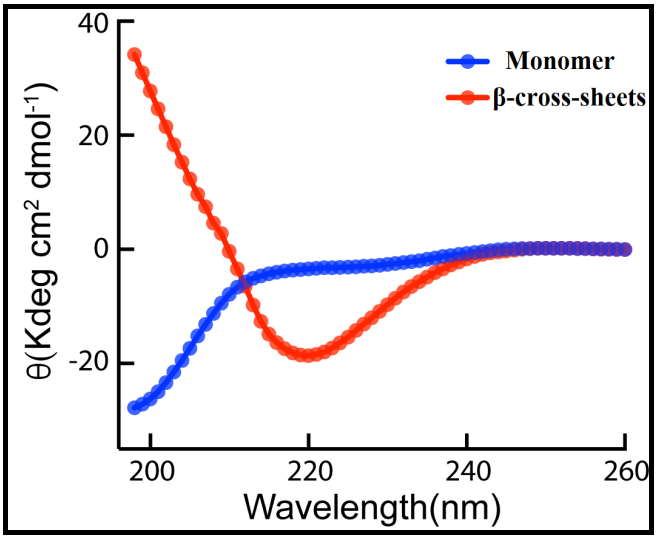

Fig. 1: CD spectra analysis of the two different forms (monomer and β-cross-sheet α-synuclein) secondary structure of proteins

CD spectra showed single negative minima at ~200 nm for random coil structure and single minima at ~218 nm for β-sheet structure spectroscopy, confirming the structure β-cross-sheet and monomeric α-synuclein protein structure obtained from Indian Institute Technology, Mumbai (Fig. 1).

Competitive NC-FLISA

For the competitive NC-FLISA, a range of β-cross-sheet antigen concentrations, spanning from 1000 ng to 0.001 ng, was assessed. These antigens were pre-incubated with fluorophore-tagged pAb in separate tubes before being introduced to the pre-coated NC discs. As illustrated in Fig. 2, the experimental findings confirm that an increase in the concentration of competitive antigen results in a corresponding decrease in fluorescence within the NC membrane. These results firmly establish the method's applicability for sample analysis, and reinforce its suitability for quantitative assessments.

In vitro validation assay to assess the applicability of pAb

To assess the utility of the pAb competitive NC-FLISA assay for detecting β-cross-sheet α-synuclein, we proceeded to investigate its reactivity using supernatants from a rotenone-induced PD model in SH-SY5Y cells at two distinct time points (24 h and 48 h). As depicted in Fig. 3, the results from this evaluation demonstrate that, at least for the analysed samples, the assay effectively detected β-cross-sheet α-synuclein. Statistical analysis utilizing nested one-way ANOVA yielded significant results (P value < 0.05).

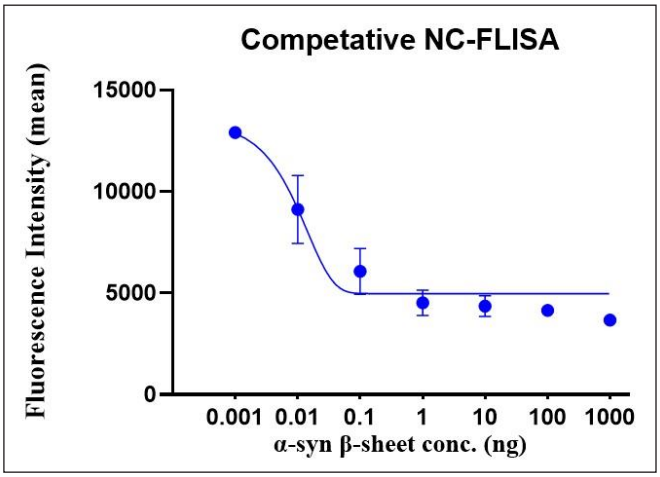

Fig. 2: Competitive NC-FLISA using β-cross-sheet α-synuclein and developed pAb

The disparity in relative fluorescence between the 24 h and 48 h time points can be ascribed to the dynamic nature of the cellular response initiated by rotenone-induced PD. After 24 h, the cellular milieu is likely in the initial stages of reacting to the pathological conditions induced by rotenone, resulting in a limited presence of misfolded β-cross-sheet α-synuclein. Consequently, the competitive assay detects a higher concentration of the target antigen, yielding elevated fluorescence.

Conversely, by the 48 h time point, the cellular response may have advanced, potentially involving mechanisms that augment the production of misfolded proteins. This could lead to an increase in the concentration of β-cross-sheet α-synuclein in the supernatant and, consequently, a decrease in fluorescence during the competitive assay.

These experimental findings validate a direct relationship between the concentration of competitive antigen in the cell supernatant and the corresponding decrease in fluorescence within the NC membrane. This inverse correlation signifies that fluorescence is

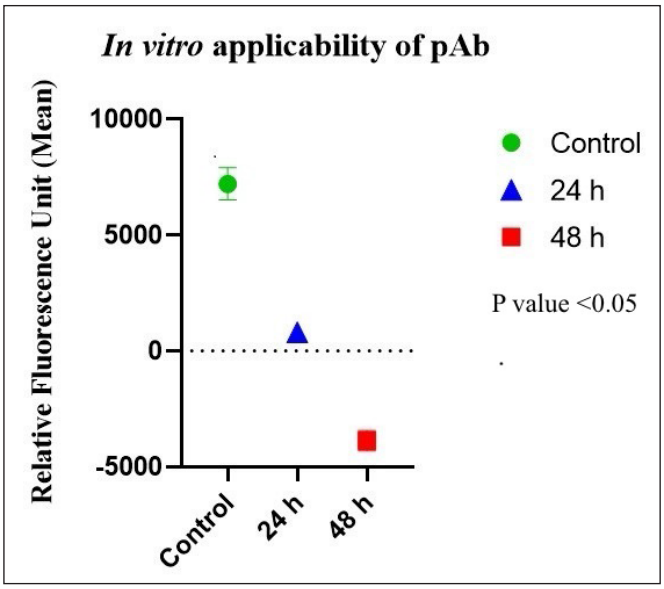

Fig. 3: Competitive NC-FLISA assay for detecting β-cross-sheet α-synuclein in *in vitro* sample

inversely proportional to the presence of β-cross-sheet α-synuclein in the sample. Notably, the control group exhibited maximal fluorescence, indicating the absence of misfolded proteins. Conversely, as the incubation time increased, fluorescence decreased, indicative of an elevated presence of misfolded proteins in the supernatant.

DISCUSSION

One of the pivotal aspects of our research revolves around the validation of our innovative Fluorescence-Linked Immunosorbent Assay (FLISA) for the detection of β-cross-sheet α-synuclein, a crucial marker in PD. The competitive NC-FLISA analysis performed here reaffirms the robustness of our assay, pinpointing the optimal antigen concentration for coating and underscoring its suitability for quantitative assessments. Moreover, our investigation highlights the effectiveness of this assay in identifying β-cross-sheet α-synuclein, as evidenced by its successful application in a rotenone-induced PD model. This validation of its sensitivity and potential diagnostic significance emphasizes the assay's potential as a valuable instrument for advancing PD research and facilitating diagnosis. This sets the stage for further exploration in this area.

The findings yield promising implications. The competitive NC-FLISA assays, employing β-cross-sheet α-synuclein as a target antigen along with our specially developed pAb, exhibit an impressive ability to discern the presence of misfolded α-synuclein within SH-SY5Y cells. This observation strongly supports the practical application of our FLISA in real-world scenarios, where detecting β-cross-sheet α-synuclein holds paramount importance for early PD diagnosis.

Furthermore, the quantitative analysis, guided by the fluorescence signals in the FLISA, establishes a direct correlation between the concentration of competitive antigen (β-cross-sheet α-synuclein) in the cell supernatant and the corresponding decrease in fluorescence within the Nitrocellulose (NC) membrane. This inverse relationship provides robust evidence that the FLISA is both sensitive and accurate in identifying β–cross-sheet α-synuclein levels, even within the complex milieu of cellular samples.

Significantly, our assays reveal that the control group exhibits maximal fluorescence, indicating the absence of misfolded proteins. Conversely, as incubation time increases, fluorescence diminishes, signifying an elevated presence of misfolded proteins in the supernatant. This finding bears particular significance in the context of PD, where early detection of β-cross-sheet α-synuclein could offer a crucial window for intervention before substantial neuronal damage ensues. Additionally, FLISA's time-saving attributes have facilitated our research progress, ultimately propelling us closer to potential breakthroughs in PD diagnosis.

CONCLUSION

Our study introduces a novel approach to address the diagnostic challenges posed by PD. By targeting the β-cross-sheet form of α-Synuclein and utilizing a non-invasive sampling approach, we have laid the foundation for a potentially transformative method for early PD detection. *In vitro* assay utilizing rotenone-induced PD in SH-SY5Y cells represents a critical step in validating the practicality and reliability of our FLISA-based approach for the early detection of PD. The successful application of FLISA in this biologically relevant model underscores its potential as a valuable tool for advancing our understanding of PD pathogenesis and, more importantly, for facilitating early diagnosis, which is pivotal for effective disease management and intervention. Our findings contribute to the broader effort of improving patient outcomes by enabling timely intervention and therapeutic strategies in the early stages of neurodegenerative disorders.

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