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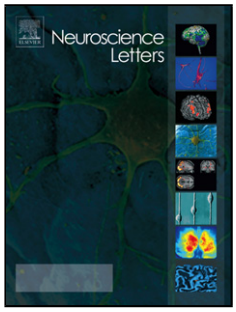

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Effects of different isoforms of apoE on aggregation of the α-synuclein protein implicated in Parkinson's disease

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Highlights

- ThT assay demonstrates aggregation of α-synuclein is influenced by apolipoprotein E
- Sandwich ELISA indicates the observed α-synuclein aggregates were multimeric in nature
- Low levels of apoE stimulate α-synuclein aggregation while higher level supresses
- Among the different isoforms tested apoE4 has the greatest stimulatory effect
- Apolipoproteins may have a role in pathogenesis of Parkinson's disease by influencing the aggregation of α-synuclein

Abstract

Parkinson's disease is a progressive brain disorder due to the degeneration of dopaminergic neurons in the substantia nigra. Parkinson's disease is a progressive brain disorder due to the degeneration of dopaminergic neutrons in the SN. The accumulation of aggregated forms of α-synuclein protein into Lewy bodies is one of the characteristic features of this disease although the pathological role of any such protein deposits in causing neurodegeneration remains elusive. Here, the effects of different apolipoprotein E isoforms (apoE2, apoE3, apoE4) on the aggregation of α-synuclein *in vitro* were examined using thioflavin T assays and also an immunoassay to detect the formation of multimeric forms. Our results revealed that the aggregation of α-synuclein is influenced by apoE concentration. At low concentrations of apoE (<15 nM), all of the isoforms were able to increase the aggregation of α-synuclein (50 μM), with apoE4 showing the greatest stimulatory effect. This is in contrast to a higher concentration (>15 nM) of these isoforms, where a decrease in the aggregation of α-synuclein was noted. The data show that exceptionally low levels of apoE may seed α-syn aggregation, which could potentially lead to the pathogenesis of α-synuclein-induced neurodegeneration. On the other hand, higher levels of apoE could potentially lower the degree of α-synuclein aggregation and confer protection. The differential effects noted with apoE4 could explain why this particular isoform results in an earlier age of onset for Parkinson's disease.

Abbreviations: PD, Parkinson's disease; Aβ, β-amyloid; CSF, cerebrospinal fluid ; R-α-syn, recombinant α-synuclein; ThT Thioflavin T; TRF, Time-resolved fluorescence

Keywords: Parkinson's disease; Lewy body; α-synuclein; apolipoprotein E; aggregation

Introduction

Parkinson's disease (PD), a progressive neurodegenerative disorder that impairs movement, and affects over 6 million people globally. Neuropathological features of idiopathic PD, the most common form of Parkinson's, include the presence of Lewy bodies (LBs) and the loss of dopaminergic neurons from the substantia nigra (SN) in the surviving neurons [1]. LBs are intracellular inclusion bodies that contain fibrils composed of a protein called α-synuclein (α-syn) [1-3]. Pathological changes involving α-syn are also found in more widespread areas of the brain in dementia with Lewy bodies (DLB) and in glial cells in multiple system atrophy (MSA) [3]. Duplication, triplication and mutation of the *SNCA*, the gene encoding α-syn, are all causes of hereditary forms of either PD or DLB [4]. The over expression of α-syn in transgenic animals produces a phenotype resembling PD, with SN degeneration, movement problems, and responsiveness to therapy with the drug L-dopa, which is still the mainstay of treatment for patients with PD [5,6]. The secondary structure and aggregation state of α-syn vary in response to its environment. In aqueous solution, α-syn has been reported to exist as a 'natively unfolded' protein, while contact with acidic phospholipids induces transformation into a structure containing α-helices [7]. Most notably, the protein present in LBs is found in the form of insoluble fibrils that contain a high content of β-pleated sheet structure [1-3]. Moreover, prefibrillar forms of α-syn often referred to as 'soluble oligomers' are toxic to nerve cells [8, 9]. These observations suggest that α-syn plays a pivotal role in the development of PD and the other 'α-synucleinopathies'.

Apolipoprotein E (apoE), the most abundant apolipoprotein in the human brain, acts to maintain cholesterol homeostasis. It is 299 amino acids in length, and exists in three different isoforms, based on the amino acid residues present at positions 112 and 158. These are apoE2 with Cys at both positions, apoE3 with Cys-112 and Arg-158, and apoE4 with Arg at both positions [10]. The presence of one or more apoEε4 alleles increases the risk of developing late-onset Alzheimer's disease, reduces age of onset, and increases cerebral β-amyloid (Aβ) plaque load [11]. ApoE has also been reported to enhance amyloid fibril formation *in vitro* by binding to Aβ, and to increase the phosphorylation of tau and promote neurofibrillary tangle formation, with apoE4 consistently showing the greatest effect [12-14]. ApoEε4 is also a risk factor for other neurological disorders, such as multiple sclerosis [15-17]. A possible role for apoE in PD is less well documented, but apoE is present in neurons and LBs of the SN in both PD and incidental

Lewy body disease (iLBD) (a possible precursor to PD) along with its receptor, low-density lipoprotein receptor-related protein 1 (LRP1), which is over expressed in these conditions [18]. ApoE is considered to have a neuroprotective role by preventing apoptosis in neurons and the anti-apoptotic mechanism is initiated when apoE binds to LRP1 [19]. Thus alterations in lipoprotein homeostasis could be an early event during the pathogenesis of PD and apoE could have a positive impact on the survival of SN neurons. On the other hand, it has been reported that deletion of apoE decreases α-syn aggregation in the brains of transgenic mice and boosts overall survival [20] suggesting that high levels of ApoE concurrent with high levels of aggregated α-syn may be a risk factor for PD. It has also been noted that PD patients who carry at least one apoEε4 allele are more vulnerable to the development of dementia than non-carriers [21, 22]. PD patients with apoEε4 also have an earlier age of disease onset [23, 24].

Although the reports mentioned above have suggested possible links between apoE, α-syn and PD, there are no previous publications examining the direct effects of apoE on aggregation of α-syn *in vitro*. Here, we have monitored the aggregation of α-syn over time in the presence of different amounts of each of the isoforms of apoE. The results clearly show that low concentrations of apoE stimulate α-syn aggregation, whereas higher concentrations cause inhibition. Amongst the different apoE isoforms, low concentrations of apoE4 were found to have the greatest effect on stimulation of α-syn aggregation.

Materials and Methods

The different isoforms of ApoE were supplied by Sigma (apoE2; SRP4760, apoE3; SRP4696 and, apoE4; A3234). Biotinylated C211 was prepared using a Sigma biotinylation kit (KB101), following the instructions of the manufacturer. Other materials included anti-α-synuclein (C211) mouse monoclonal antibody (Santa Cruz, sc-12767), anti-α-synuclein Syn 1 mouse monoclonal antibody (BD Tranduction Labs, 610787) and MonoQ 5/50 GL and Superdex 200 10/300 columns (GE healthcare).

Production of recombinant α-synuclein protein

Recombinant α-syn (R-α-syn) protein was produced by using a pET11a expression vector introduced into *E.Coli*. An agar plate containing chloramphenicol and carbenicillin (CM+CB) was streaked with pET11a α-syn glycerol and incubated overnight at 37 °C. A colony was

transferred to a tube containing 5 ml Luria-Bertani broth (CM+CB) and incubated overnight. 1 ml of overnight culture was transferred into 100 ml LB broth (CM+CB) and incubated overnight at 37 °C. 800 ml of LB broth (CM+CB) were placed into a large shaking incubator, at 200rpm and left to reach 37 °C for about 20 min. Then, 50 ml of overnight culture were added into each flask and the OD600 measured approximately every 30 min until it reached ~0.5. This was followed by induction with IPTG for 2 h at 37 °C. After 2 h of incubation, the cultures were centrifuged at 3000 x g for 30 min at 4 °C. The supernatant was discarded and each pellet was resuspended in 10 ml of cell suspension buffer, pooled together, and stored frozen at -80 °C. Resuspended pellets were thawed in a 37 °C water bath, but were not allowed to reach 37 °C. The warmed tube was placed on ice and its contents were sonicated for 6×20 sec bursts, with 20 sec intervals. After the second sonication, 10 µl of PMSF was added per 1 ml of culture. The samples were transferred into 8 ml glass ultracentrifuge tubes and centrifuged at ~50,000 g for 2 hrs at 4 °C to separate the lysate containing R-α-syn from *E.Coli* proteins. 500 µl of *E. Coli* lysate expressing α-syn was applied to the MonoQ 5/50 GL column and bound proteins were eluted with a linear gradient of KCl (0 to 0.5 M) at a flow rate of 0.5 ml/min. Protein concentration in each fraction was measured by absorbance at 280 nm. Fractions containing α-syn , detected by SDS-PAGE gel, were pooled and further purified on a gel filtration column (Superdex 200 10/300). The purity of the final product was estimated as >90 % by SDS-PAGE and immunoblotting.

Thioflavin T assay

A ThT stock solution (2.5 mM) was prepared by adding 8 mg ThT to 10 mL phosphate buffer (10 mM phosphate, 150 mM NaCl, pH 7.0), filtered through a 0.2 µm syringe filter, and stored at 5 °C in a foil-wrapped tube for not longer than one week. For each experiment, the ThT stock solution was diluted with phosphate buffer to generate the working solution (0.05 mM). In the 'continuous' ThT assay, 15 µl of ThT working solution was mixed with R-α-syn solution and the fluorescence intensity of ThT was monitored by excitation at 440 nm and emission at 482 nm over a 5 day incubation period at intervals of 10 mins. In the 'stopped' ThT assay, the R-α-syn solution was incubated at 37 °C and samples were removed after set incubation time periods prior to the addition of 50 µl of ThT to each sample. The intensity of ThT fluorescence was then measured as before. The mean % aggregation was calculated relative to R-α-syn alone

Immunoassay for measuring oligomeric/polymeric α-synuclein

An ELISA plate was coated by overnight incubation with 1 µg/ml of non-biotinylated anti-α-syn C211 antibody, diluted in 200 mM $NaHCO_3$, pH 9.6, containing 0.02% (w/v) sodium azide, at 4 °C overnight. The wells were then washed 4 times with PBS-T and incubated for 2 h at 37 °C with 200 µl/well of freshly prepared blocking buffer. The plate was washed again with PBS-T, and 100 µl of the sample in each well incubated at 37 °C for 2 h. After washes with PBS-T, 100 µl/well of biotinylated C211 diluted to 1 µg/ml in blocking buffer was added and incubated at 37 °C for 2 h. The plate was washed 4 times with PBS-T and incubated with 100 µ/well of streptavidin-europium for 1 h at RT on a shaker. The plate was washed 4 times with PBS-T and 100 µl of europium enhancer (PerkinElmer Life Sciences Inc. USA) added to each well and the plate incubated for 10 min on a shaker. The time-resolved fluorescence (TRF) assay was performed by using the standard conditions for detection of europium fluorescence (excitation at 340 nm, and fluorescence emission at 615 nm) in a Perkin Elmer Wallac Victor2^{TM} plate reader.

Data analysis

Data are expressed as mean ±SEM, analysed by one-way ANOVA to evaluate the differences between group means. Then, Tukey's HSD method, as a single-step multiple comparison procedure, was used in conjunction with ANOVA (XLSTAT product) to find the mean levels of α-syn aggregation that are significantly different from each other at the four concentrations of each of the different apoE isoforms (p-value < 0.01)

Results

Anion exchange chromatography was effective in the initial purification of the cell lysate to yield R-α-synuclein as the main product (figure 1a) seen on a coomassie blue stained SDS-PAGE Gel. A further purification step using size exclusion chromatography improved the purity of the final R-α-syn to homogeneity as indicated by a single band (figure 1b) on a silver stained SDS-PAGE Gel. Immunoblotting (figure 1c) using two different α-synuclein specific monoclonal antibodies confirmed the identity.

Two different thioflavin T (ThT) assays were employed to monitor the aggregation of α-syn in the presence of each of the different apoE isoforms. For the 'continuous' assay, R-α-syn, apoE and ThT were simultaneously present throughout the assay, while in the 'stopped' assay R-α-syn

was preincubated with apoE prior to addition of ThT for recording fluorescence intensity. This 'stopped' assay method was used to determine whether the presence of ThT has any influence on α-syn aggregation. Statistical analysis was performed using Tukey's HSD method.

In the continuous ThT assay, the lower concentrations of apoE tested (1, 5 and 15 nM) consistently increased the aggregation of α-syn (which was present at 50 μM) whereas the highest concentration of apoE (50 nM) inhibited α-syn aggregation (Figure 2). In all cases, the aggregation typically proceeded (Figure 2a) with a time delay of at least 24 h, reflecting the need for a nucleation process. This was followed by biphasic behaviour, exhibiting a slow aggregation phase in the next 36 h eventually leading to a rapid aggregation process at 60-80 h. The length of the nucleation phase was not greatly affected by the presence of apoE, suggesting that apoE does not have any major influence on the onset of α-syn aggregation. The main effect of apoE was to enhance or inhibit the level of the ThT fluorescence signal seen towards the end of the incubation period, which reflects the final number of fibrils formed. The aggregation process approached completion by 120 h, which was taken as the end point. Figure 2b presents the percentage of α-syn aggregation (relative to R-α-syn alone) at the end of this 120 h incubation, in the presence of the different apoE isoforms. A small differential effect was seen for the different isoforms, with the lowest concentration of apoE4 (1 nM) having the greatest stimulatory effect on α-syn fibril formation.

In the stopped ThT assay, the ThT signal seen at the end of the time course (120 h), reflecting the amount of α-syn fibrils formed, was increased in the presence of low concentrations of apoE (15 nM or less) and decreased at the highest apoE concentration (50 nM) (Figure 3). The kinetics of the aggregation in the absence of ThT exhibited a similar biphasic profile to that observed in the presence of ThT for all of the isoforms, as illustrated by apoE2 (Figure 3a). Analysis of the final percentage (at 120 h incubation) of α-syn aggregation (figure 3b) in the presence of the different apoE isoforms, relative to R-α-syn alone, confirms that apoE4 has the greatest stimulatory effect on α-syn aggregation at 1 nM apoE concentration. As can be seen in Figures 2 and 3, apoE4 at 1 nM concentration significantly increases the aggregation of α-syn relative to the other isoforms in both types of ThT assay.

In an attempt to verify these results, an alternative method was employed to monitor α-syn aggregation. The same samples from the stopped ThT assay were also analyzed by an immunoassay technique for determination of multimeric α-syn. This involves capture of α-syn aggregates with C211 anti-α-syn antibody followed by detection with a biotinylated form of the same antibody. Monomeric α-syn does not give a signal in this assay [25]. The data from this experiment are presented in Figure 4, which also includes an overall summary (figure 4d) of the effect of each apoE isoform. The results from this assay were very similar to those obtained with both of the ThT assays. The kinetic profiles (Figure 4a-c) were also comparable to those observed with both of the ThT assays (Figure 2 and 3). The ThT assay specifically detects β-pleated structures, whereas the sandwich immunoassay is not expected to distinguish between different secondary structures present in the aggregate but selects for any of the multimeric forms. At low apoE concentrations, the rank order (figure 4d) for stimulation of α-syn aggregation was always apoE4>apoE3>apoE2.

All of the data from these three assays are consistent with each other and confirm that apoE4 has the greatest stimulatory effect on α-syn aggregation, at low apoE concentrations. Tukey's HSD test confirms (Figures 2b, 3b, and 4d) that the stimulatory effect of apoE4 is always significantly greater than that of the other isoforms at 1 nM (indicated by the different letters 'a' and 'b'), but the effects of the apoE isoforms are not always significantly different from each other at the higher concentrations (marked by bars with the same letter 'a'). The average of the percentage α-syn aggregation over all three assays, as determined at the end of the incubation time course, has been plotted for each apoE concentration (Figure 5). Here, the data for apoE2, apoE3 and apoE4 have also been combined, so that the overall effects of different concentrations of apoE on α-syn aggregation can be appreciated. The results clearly show that low concentrations of apoE stimulate α-syn aggregation, whereas the highest concentration causes some inhibition.

Discussion

In this study, we examined the effects of each isoform of apoE on the aggregation of recombinant α-syn (R-α-syn) *in vitro*. For this purpose, we established two different ThT assays (for detection of β-pleated sheet fibrils) and an immunoassay (for detection of multimeric α-syn). The data from all these assays were in remarkably close agreement, and show that low

concentrations of apoE increase the amount of late-stage aggregates formed, with apoE4 having the greatest effect, in contrast to higher concentrations of apoE, which inhibit α-syn aggregation. One possible explanation for these effects of apoE is that it can also self-aggregate, with apoE4 doing so at a faster rate than the other isoforms, and apoE2 aggregating the slowest [26]. If the aggregated form of apoE acts as a 'nucleus' for α-syn aggregation, this could explain why apoE4 appears to enhance aggregation to a greater degree than the other isoforms. Under appropriate conditions, apoE could self-aggregate, or co-aggregate with α-syn, and so act as a 'seed' for other α-syn molecules to become incorporated into the resulting β-sheet aggregates. However, this idea conflicts with the fact that apoE self-aggregation is more closely related to α-helical interactions than the formation of β-sheet structures [26] and so might not be expected to 'seed' β-sheet formation. Also, this type of mechanism cannot easily explain why the highest concentration of apoE actually inhibited α-syn aggregation.

It is also possible that α-syn interacts directly with apoE, with the three isoforms of the latter having differential binding properties, and this could also explain the effects seen on α-syn aggregation. It is interesting to note that apoE has been reported to bind directly to intermediate aggregates or 'oligomers' of the β-amyloid peptide (Aβ) associated with Alzheimer's disease, with apoE4 having the greatest affinity for such binding [27]. Given the fact that Aβ and α-syn both have the ability to form highly similar β-pleated sheet structures, which can even be recognised by the same monoclonal antibody [9], it would not be surprising if apoE also had the ability to bind to oligomeric α-syn. The kinetic profiles follow a similar pattern for all three assays, and so it can be argued that the aggregation predominately results in the formation of β-pleated sheet structures. Furthermore, as the onset of aggregation was detected at similar time points for all three assays, it could be inferred that in the nucleation phase any small seed structures present must be loosely associated monomeric forms of α-syn and free of the more strongly associated β-pleated sheet.

Among the different apoE isoforms, low concentrations of apoE4 were found to stimulate α-syn aggregation to a greater extent than the other isoforms. This effect of apoE4 on α-syn aggregation could help to explain why apoEε4 is a risk factor for earlier onset of PD [24], which certainly warrants further investigation.

Taken together, the results from this study have revealed that a possible direct molecular interaction between soluble α-syn and various apoE isoforms can influence the extent of α-syn

aggregation *in vitro*. A close agreement between the ThT assays and the results from the immunoassay underpins the fact that the α-syn species being detected were oligomeric in nature. Such oligomeric forms are well reported to be neurotoxic [28]. Post-mortem ventricular CSF of individuals [32], without evidence of dementia or parkinsonism, has an apoE content of around 6.9 µg/ml compared to a lower value of 5.7 µg/ml for PD group. The highest concentration (50 nM) we tested is between 3 to 4 to fold below such levels but caused suppression of α-synuclein aggregation. However in the lower nM range (<50 nM) the aggregation was stimulated indicating unusually low levels of ApoE may potentially contribute in early seeding of the deposition of oligomeric forms of the α-synuclein. Lower apoE levels in the CSF of a PD animal model relative to normal animals have been documented [33]. In a transgenic mouse model of α-synuclein the deletion of apoE expression also decreased the levels of insoluble, in favour of soluble, α-synuclein and slowed the neurodegeneration [20]. The absolute intraneuronal levels of apoE in PD group are probably a more important consideration but unknown. The mechanism of how apoE, at very low levels, could contact the cytoplasmic α-synuclein is obscure. At present, evidence is lacking in the literature to support the view that the α-syn and apoE can interact directly *in vivo*. One of the main reasons for this is the fact that α-syn has been thought historically to be a cytoplasmic protein, whereas apoE is extracellular. However, it is now clear that extracellular forms of α-syn do exist [29], and could be involved in a 'prion-like' seeding mechanism for the spread of α-syn pathology to adjacent parts of the brain [30]. Thus extracellular interactions between α-syn and apoE could influence the aggregation of the former, and have an effect on PD pathogenesis. Also, it is feasible that an internalisation mechanism for apoE may bring the two molecules together. In this context, it is interesting to note that altered neuronal apoE trafficking has been implicated to occur in PD and is speculated to be involved in α-syn deposition [18]. Many fundamental questions remain unanswered at this stage due to uncertainties in mechanism of action for the toxicity of oligomeric α-synuclein and technological challenges in studying α-syn aggregation at intracellular level [31]. In our findings, while low levels of apoE aggravated the extent of α-syn aggregation, the higher level tended to supress aggregation. Neurodegeneration induced by overexpressed α-syn in transgenic mice also caused higher levels of apoE [20]. Could this be the body's natural defence to reduce aggregation and hence help combat PD?

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Figure legends

Figure 1. (A) Coomassie brilliant blue stained SDS-PAGE gel of the anion exchange purified cell lysate. (B) Silver stained SDS-PAGE gel of the purified final R-α-Synuclein. Western blots of the final product using anti-α-syn syn1 (C) and anti-α-syn C211 (D) monoclonal antibodies.

Figure 2. α-syn (50 μM) aggregation curves recorded in the presence of ThT at different concentrations of apoE2. The curves at time point 120 hrs reading from top to bottom represents apoE concentrations of 1, 5, 15, 0, and 50 nM. The very bottom curve represents background signal without any apoE and α-synuclein.

A. Percentage of end-stage α-syn aggregation seen in the presence of different apoE isoform concentrations. The α-syn (50 μM) was incubated for 120 h in the presence of both ThT and each given concentration of apoE. Data show mean +/- SEM (n = 3) percentage aggregation, relative to α-syn alone. Within each defined concentration of apoE, means with different letters are significantly different (Tukey's HSD, $p<0.01$).

Figure 3. α-syn (50 μM) aggregation curves at indicated concentrations of apoE2. ThT was added after incubation of the α-syn with apoE. The curves at time point 120 hrs reading from top to bottom represents apoE concentrations of 1, 5, 15, 0, and 50 nM.

A. Percentage of end-stage α-syn aggregation seen in the presence of different apoE isoform concentrations prior to adding ThT. The α-syn (50 μM) was incubated, with given concentration of apoE, for 120 h in the absence of ThT. Data show mean +/- SEM (n = 3) percentage aggregation, relative to α-syn alone. Within each defined concentration of apoE, means with different letters are significantly different (Tukey's HSD, $p<0.01$).

Figure 4. (A-C) Effect of apoE isoforms at different concentrations of (A) apoE2, (B) apoE3 and (C) apoE4 on the kinetics of 50μM α-synuclein aggregation using sandwich capture immunoassay. Europium was used for TRF measurements. The curves at time point 180 hrs from top to bottom represents apoE concentrations of 1, 5, 15, 0, and 50 nM.

(D) Percentage of end-stage α-syn aggregation seen in the presence of different apoE isoform concentrations in the immunoassay for multimeric ThT. The α-syn (50 μM) was incubated for 120 h in the presence of each given concentration of apoE. Data show mean +/- SEM (n = 3) percentage aggregation, relative to α-syn alone. Within each defined concentration of apoE, means with different letters are significantly different (Tukey's HSD, $p<0.01$).

Figure 5. Percentage of end-stage α-syn aggregation seen in the presence of different concentrations of apoE. The data presented in Figures 1b, 2b and 3d have been averaged over all three apoE isoforms, and over all three assays. Data are plotted as mean +/- SEM. Across the different concentrations of apoE, means with different letters are significantly different (Tukey's HSD, $p<0.01$).

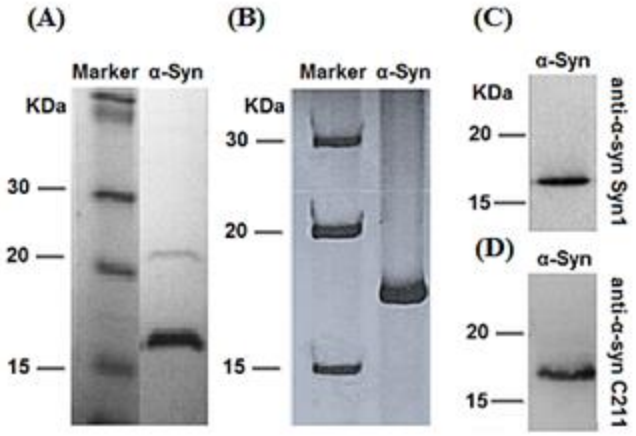

Fig. 1

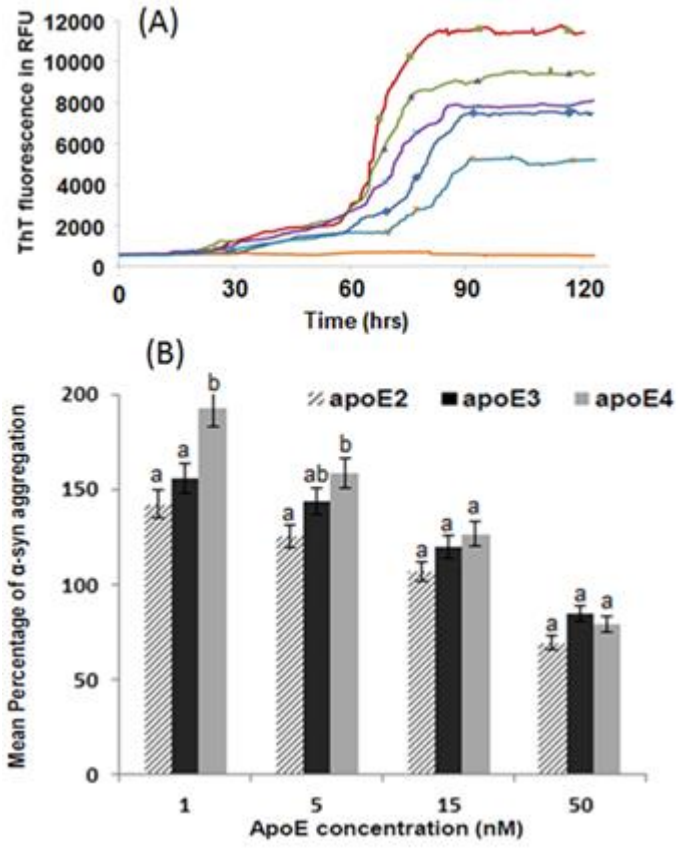

Fig. 2

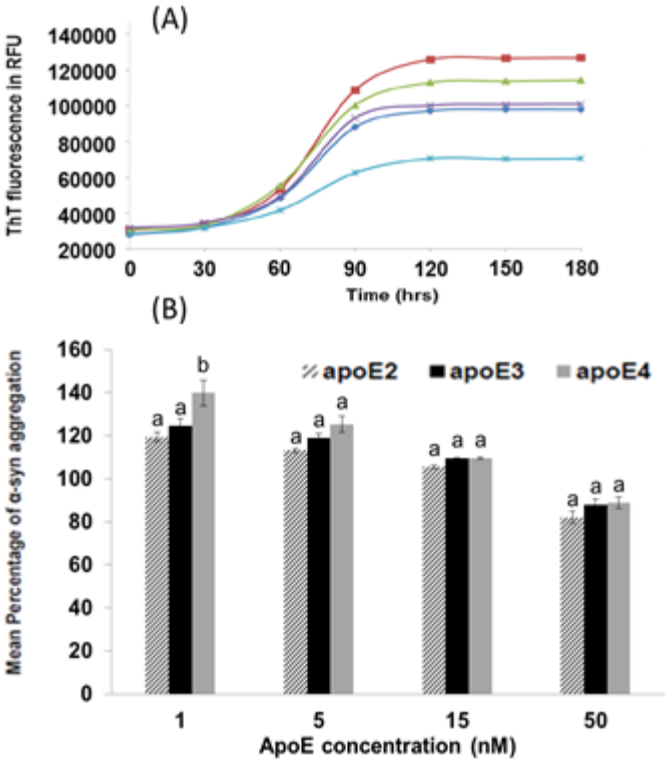

Fig. 3

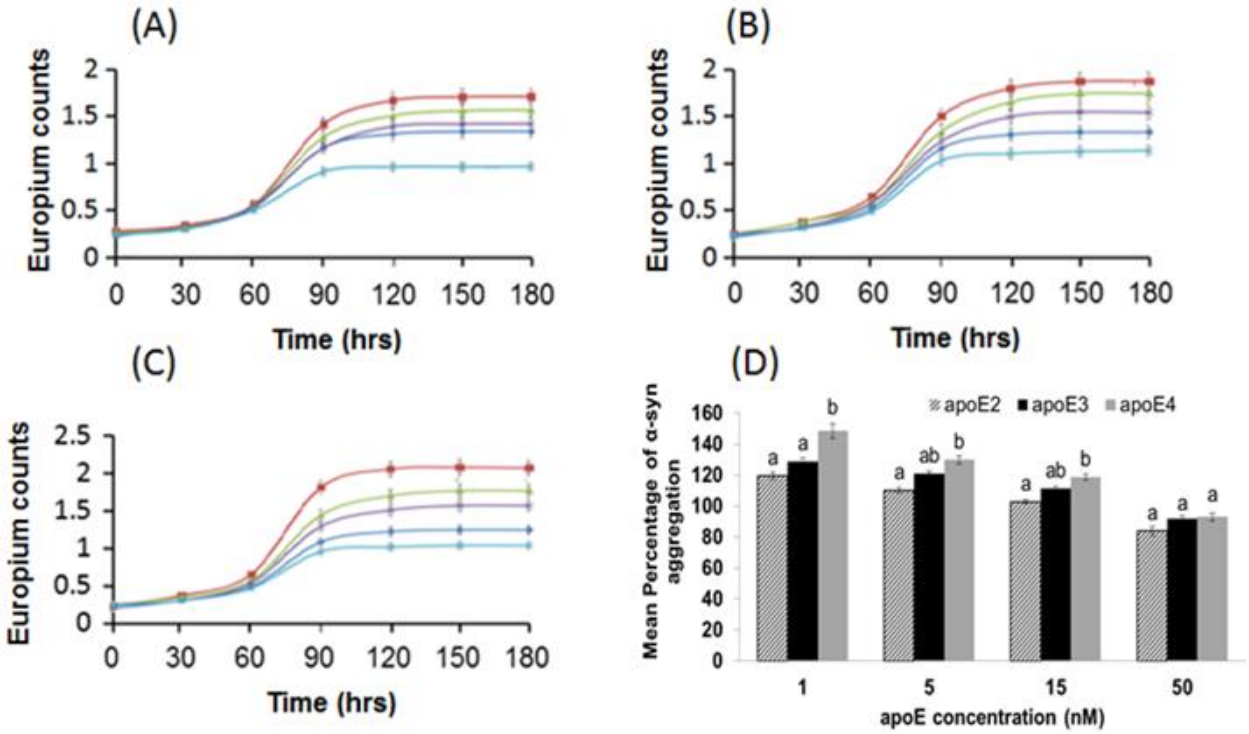

Fig. 4

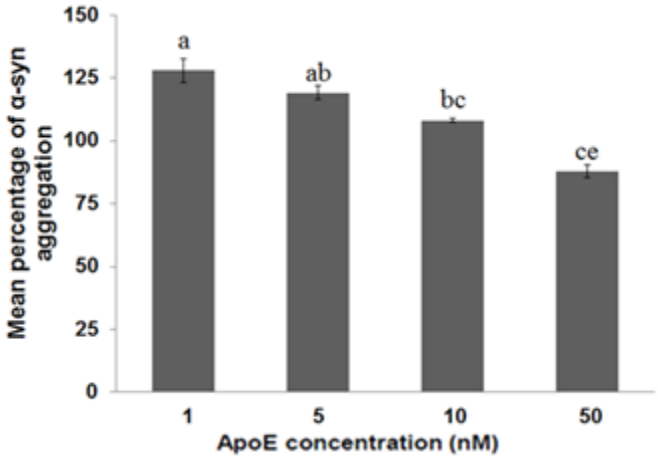

Fig. 5