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# Poly-γ-glutamic acid alleviates cytotoxicity and inflammation induced by pre-formed fibrils of α-synuclein in murine primary astrocytes

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ABSTRACT

Poly-γ-glutamic acid (γ-PGA) is a bacterial-derived natural biopolymer that has gathered significant interest due to its antioxidant, anti-inflammatory, and neuroprotective properties. These characteristics make γ-PGA a potential candidate for the treatment of neurodegenerative diseases. In Parkinson's disease (PD), whose key pathological feature is the accumulation of neuronal α-synuclein aggregates, astrocytes, in addition to microglia, play a crucial role in clearing these aggregates; however, their capacity is limited. Overwhelmed astrocytes trigger an inflammatory response that exacerbates neurodegeneration. Therefore, strategies aimed at regulating the uptake of extracellular α-synuclein aggregates by astrocytes and mitigating inflammation could hold therapeutic promise. This work aimed to investigate the potential of γ-PGA in preventing or reversing the toxicity and inflammatory response induced by pre-formed α-synuclein fibrils (PFFs) in murine cortical astrocytes. Cell viability assays demonstrated that γ-PGA can counteract the toxicity induced by α-synuclein PFFs. Confocal microscopy and 3D reconstruction analyses revealed that γ-PGA colocalizes with PFFs, leading to a reduction in the uptake of these aggregates by astrocytes and a subsequent decrease in their inflammatory response. Consequently, γ-PGA emerges as a promising candidate for further investigation in the therapeutic management of PD.

## 1. Introduction

Poly-γ-glutamic acid (γ-PGA) is a biodegradable, non-toxic, eco-friendly, and non-immunogenic biopolymer whose interest in biomedicine has seen recent development. γ-PGA consists of glutamic acid monomers that are coupled to each other *via* amide bonds between α- or γ-carboxylic groups [1]. Unlike conventional proteins, γ-PGA is synthesized through the Poly-γ-Glutamate Synthetase intermembrane complex that allows the producer to form γ-peptidic bonds and adjust the molar mass [2]. Among its peculiar features, γ-PGA has

*Abbreviations:* FITC, Fluorescein-5-isothiocyanate; HBSS, Hanks' balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PD, Parkinson's disease; PFFs, pre-formed α-synuclein fibrils; RT, room temperature; ThT, Thioflavin T; TLR4, Toll-like receptor 4; γ-PGA, Poly-γ-glutamic acid.

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demonstrated anti-inflammatory and antioxidative properties and has been shown to alleviate neuronal cell death and memory deficits [3–6], highlighting its potential therapeutic value in neurodegenerative diseases.

Parkinson's disease (PD) is the most common neurodegenerative movement disorder [7,8], pathologically associated with neuronal cell death in certain brain regions, and with the presence of aggregated α-synuclein-rich intraneuronal inclusions, known as Lewy bodies and Lewy neurites [9]. The α-synuclein aggregation process is known to result in the formation of various pathological species, culminating in the appearance of fibrils, the main component of such inclusions [10]. Current evidence also suggests that aggregates of α-synuclein may be transmitted from cell to cell, contributing to disease progression [11]. Several studies in cellular models support the idea that such transfer could occur through different pathways, including tunneling nanotubes, endocytosis, and exosomes [12–14]. Moreover, it is known that α-synuclein aggregates can be secreted from neurons during stress or their degeneration, resulting in an increased concentration of aggregates in the extracellular environment [15,16]. Extracellular α-synuclein aggregates not only could be taken up by neurons but could also affect the phenotype and behaviour of other cell types such as astrocytes [17–19]. Indeed, astrocytes are involved in the clearance of extracellular aggregated α-synuclein species released from neurons by internalizing and degrading them through the endo-lysosomal pathway, thus playing a protective role for neurons. However, their capacity is limited, and excessive uptake can overwhelm astrocytes, leading to cellular toxicity [20]. Moreover, high concentrations of α-synuclein in the extracellular environment activate the innate immune-dependent inflammatory pathway that induces the acquisition by astrocytes of a reactive inflammatory phenotype (A1 type). This feature could contribute to the progression of the pathology as astrocytes lose the ability to promote neuronal survival, synaptogenesis, outgrowth, and phagocytosis, and release pro-inflammatory molecules that trigger neuronal cell death [21,22]. Thus, approaches that can regulate extracellular α-synuclein aggregates and act on neuroinflammation might be beneficial.

Here we aim to explore the biological effect of γ-PGA, produced through fermentation from generally recognized as safe (GRAS) organism *Bacillus subtilis* natto, in murine primary astrocytes exposed to α-synuclein pre-formed fibrils (PFFs), a recognized cellular model used to recapitulate astrocyte pathological hallmarks in PD [23,24]. We investigated the protective effect of γ-PGA on cell viability and cell inflammation, focusing our attention on the interplay between γ-PGA and α-synuclein PFFs. To this end, we tested two types of conditions through administration of γ-PGA before or after the treatment with α-synuclein PFFs. From these investigations, we observed that γ-PGA reverses PFF cytotoxic effect by acting on the amount of their internalization and cellular inflammation. Interestingly, *in vitro* data indicate that γ-PGA could potentially affect α-synuclein aggregation. Collectively, our data suggest that γ-PGA should be further explored as a novel therapeutic compound in the context of α-synuclein pathology and its treatment.

## 2. Material and methods

### 2.1. α-Synuclein pre-formed fibrils (PFFs) preparation

Human α-synuclein was produced by *E. coli* (BL21(BE3)) and purified as previously described [25]. Lyophilized α-synuclein was resuspended in sterile PBS, and ultracentrifuged at 220,000*g* for 45 min at 4 °C. Then, the obtained supernatant was collected and placed in the thermomixer for 14 days at 1000 rpm. Enriched-PFFs, isolated by the soluble part of the preparation by centrifugation at 16,100*g* for 15 min, were then quantified relative to the initial concentration of monomer before fibrillation and resuspended in sterile PBS at the concentration of 5 mg/ml [26]. α-Synuclein PFFs' fibrillation and structure were verified by Thioflavin T (ThT) assay and electron microscopy (Fig. S1). For ThT assay, 10 μM α-synuclein PFFs and supernatant containing monomer were added to 25 μM of ThT in PBS, mixed and incubated for 15 min at RT. 25 μM of ThT in PBS was used as a control measurement. Fluorescence emission spectra were recorded at 482 nm upon excitation at 440 nm confirming the increased fluorescence of α-synuclein PFFs, containing amyloid-like fibrils, compared to monomers (Fig. S1, A). For electron microscopy, 7 μl of 25 μM protein sample was applied to glow discharged carbon formvar copper grids and allowed to air-dry. Then, negative staining was performed with 1 % uranyl acetate, applied twice to the grids for 15 s. Electron microscopy images were captured using a Talos L120C microscopy (Fig. S1, B).

pHrodo α-synuclein PFFs were generated by using pHrodo™ iFL Green Microscale Protein Labeling kit (Thermo Fisher Scientific, P36015). In detail, α-synuclein PFFs, resuspended at a concentration of 1 mg/ml in PBS with 100 mM sodium bicarbonate, were incubated with pHrodo, dissolved in 50 μl of DMSO for 90 min in the dark. pHRodo α-synuclein PFFs, isolated by centrifugation at 16,100*g* for 15 min, were washed with PBS twice to remove the unconjugated pHrodo and resuspended in sterile PBS at the desired concentration.

### 2.2. γ-PGA preparation

Lyophilized γ-PGA (Natto, Japan) of 193 kDa molecular weight was resuspended in sterile PBS to obtain a 2 mM stock solution. Then, the initial pH of 5.5 was adjusted to 7.4.

For the visualization in immunofluorescence, γ-PGA was conjugated with Fluorescein-5-isothiocyanate (FITC) (Merck, 3326-32-7) as previously described [27]. In detail, γ-PGA was incubated with FITC with a 1:0.01 ratio in the dark at RT. After 90 min, the solution was eluted in a PD Spintrap™ G-25 column using sterile PBS (Merck, GE28–9180-04), to remove the unconjugated FITC.

### 2.3. Cortical primary astrocyte culture

Primary astrocytic cultures were derived from the spare cortex of postnatal days 1–3 (P1-P3) C57BL/6J WT mice, as previously described [28]. Animal procedures were carried out in accordance with the guidelines of the care and use of laboratory animals established by Italian and European Directives (D. Lgs n° 2014/26, 2010/63/UE). Cerebral cortices were dissociated in cold Hanks' balanced salt solution (HBSS, Euroclone) with 1 % Penicillin/Streptomycin (Euroclone). After enzymatic and mechanical dissociation, the cell suspension was centrifuged twice for 5 min at 200*g*. Successively, cells were resuspended in complete astrocytes medium containing DMEM high glucose, 10 % Fetal Bovine Serum (FBS, Euroclone), 1 % Penicillin/ Streptomycin. Cell suspension was plated in a T75 or T25 flask previously coated with poly-L-Lysine 10 μg/ml and maintained in culture at 37 °C with 5 % $CO_2$. The following day, the flasks were washed with PBS to remove any residual tissue. The culture was maintained until confluence, at which point cells were shaken at 200 rpm for 2 h to remove microglia and oligodendrocytes, obtaining a pure astrocyte culture.

### 2.4. Cell treatment

Cells were cultured onto poly-L-Lysine (10 μg/ml)-coated coverslips in a 24-well plate, in a poly-L-Lysine (10 μg/ml)-coated 48-well plate or 6-well plate at a density of $2.1 \times 10^4/cm^2$ for 24 h. Then, cells were treated with 2 μM PFFs and 27.5 μM γ-PGA in two conditions: *i*) the PRE treatment, in which γ-PGA is added before PFFs, and *ii*) the POST treatment, in which cells are first exposed to PFFs and then to γ-PGA. In both cases, PFFs treatment was carried out for 18 h while γ-PGA is added 2 h before or after PFFs.

### 2.5. Cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

(MTT) assay was performed to evaluate the cytotoxic effect of γ-PGA and PFFs. Cells were seeded in a poly-L-Lysine-coated 48 well plate, cultured for 24 h and treated with 2 μM PFFs, γ-PGA at different concentrations (0.275, 2.75, 27.5 μM) or a combination of the two following PRE and POST treatment. At the end of the treatment, cells were incubated with 0.5 mg/ml MTT solution (Merck, code number: M2128) for 3 h at 37 °C. Formazan crystals that had formed were dissolved using a 0.1 M HCl, Triton X-100 in isopropanol solution. Then, optical density (OD) was recorded at 570 nm using an Ensight plate reader.

### 2.6. Immunofluorescence

Cells were fixed with 4 % paraformaldehyde for 10 min at RT and blocked with 1 % BSA diluted in PBS containing 0.3 % Triton X-100 for 1 h at RT. Primary antibodies were diluted in 1 % BSA, 0.1 % Triton X-100 in PBS; in detail, the following antibodies, incubated overnight at 4 °C, were used: mouse anti-α-synuclein 1:500 (Merck, code number: S5566), goat anti-Complement C3 1:500 (Life technologies, code number PA1-29715), chicken anti S100β 1:700 (Synaptic Systems, code number: 284006), and anti-LAMP1 1:500 (Abcam, code number ab24170). The following day, after three washes with PBS, cells were incubated for 45 min at RT with secondary antibodies diluted in 0.1 % BSA in PBS. The secondary antibodies used were: donkey anti-mouse Alexa 647 1:500 (Life technologies, code number: A32787), donkey anti-rabbit Alexa 488 (Life technologies, code number: A21206), donkey anti-goat Alexa 488 1:300, and donkey anti-chicken 568 1:300 (Jackson Immunoresearch, code numbers: 705-545-147, and 703-165-155, respectively). Later, after 3 washes with PBS, nuclei were stained using Hoechst 33342 1:5000 in PBS for 10 min at RT and mounted using Mowiol®, DABCO®. Images were acquired with a 20× objective, an oil-immersion 60× objective or a silicon-immersion 100× objective, using a Nikon spinning disk confocal microscope, equipped with CSI–W1 confocal scanner unit, and a Nikon A1 laser scanning confocal microscope.

### 2.7. 3D reconstruction

For 3D visualization, images were imported into arivis Vision4D® 3.6.0 software (Zeiss Company). The region of interest (ROI) from 100× acquisitions was created using the "Transformation gallery > Crop" tool. The "Intensity threshold segmentation" pipeline was applied to reconstruct α-synuclein PFFs, S100β, and nuclei continuous staining. γ-PGA staining, which shows a punctate distribution, was reconstructed using the "blob finder" pipeline.

### 2.8. Western blotting

Primary astrocytes, seeded in a 6-well plate, were lysed in Laemmli buffer. Equal amounts of protein (20 μg) were separated by SDS-PAGE and transferred overnight to a PVDF membrane (Immobilon®-P transfer membrane, Merck, code number IPFL00005). Membranes were blocked with 5 % BSA diluted in Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl, pH 7.6) for 1 h at RT and incubated overnight at 4 °C with the following primary antibodies diluted in TBS containing 0.1 % Tween-20 (TBST): LC3 1:1000 (SIGMA-Aldrich, code number: L8918), p62 1:2000 (SIGMA-Aldrich, code number P0067), GAPDH 1:5000 (SIGMA-Aldrich, code number: G8795). Then, membranes were washed with TBST for 3 times and incubated with secondary antibody donkey anti-rabbit Alexa 488 1:4000 (Invitrogen, code number A21206) for 1 h at RT. After 3 washes, blots were visualized using iBright™ FL1500 (Life technologies) and band intensities were quantified using ImageJ software.

### 2.9. Enzyme linked immunosorbent assay (ELISA)

Supernatants of 200,000 cells were collected after 18 h of treatment as previously described, and analyzed for IL-6 (BioLegend, code number 431301), TNF-α (BioLegend, code number 430901), CXCL10 (Bio-Techne, code number DY466-05), and IL-1β (BioLegend, code number 432601) by ELISA according to the manufacturer's instructions. ELISA plates were read on microplate reader (SAFAS MP96), and data were analyzed with Prism software.

### 2.10. α-Synuclein aggregation studies

*In vitro* studies were performed to evaluate the potential effect of γ-PGA on α-synuclein aggregation. The experiments were performed using a black 384-well optical flat bottom plate (Life technologies). Each well was filled with the reaction mix composed of 170 mM NaCl, 40 mM PBS, pH 8, 10 μM Thioflavin-T (ThT), 7 μM α-synuclein with or without the presence of γ-PGA at three different concentrations (2.75 μM, 27.5 μM and 275 μM), reaching a final reaction volume of 50 μl. Two silica beads (0.8 mm) were added to each well to sustain α-synuclein aggregation. The capacity of γ-PGA to undergo self-assembly was evaluated. Finally, 7 μM PFFs were pre-incubated with 275 μM γ-PGA for 2 h at 37 °C to assess the effect of γ-PGA treated PFFs on α-synuclein aggregation compared to untreated PFFs. Each experimental condition was analyzed in quadruplicate to ensure reliability. The plates were inserted into a FLUOstar CLARIOSTAR microplate reader (BMG Labtech) and subjected to alternating cycles of shaking (1 min, 600 rpm, single orbital), and incubation (14 min at 42 °C). Fluorescence readings (480 nm) were taken every 15 min (30 flashes per well at 450 nm). The mean fluorescence values obtained from the four replicates of each sample were plotted on a graph against time.

Following the aggregation kinetics, samples were analyzed with transmission electron microscopy as described in Section 2.1.

### 2.11. Statistical analyses

Statistical analyses were conducted using GraphPad Prism 8 software (San Diego, CA, USA). All quantitative data are expressed as mean ± SEM and represent at least four independent sets of experiments. Depending on whether the data fitted a normal distribution, parametric or non-parametric One-way ANOVA was used for multiple comparisons of data. Significance was established at $p$ value $<0.05$.

## 3. Results

### 3.1. γ-PGA rescues α-synuclein PFF induced cytotoxicity on murine primary astrocytes

γ-PGA is a well-characterized, non-toxic, and non-immunogenic biomacromolecule [1] but its effect on murine primary astrocytes has yet to be investigated. Thus, to examine the potential cytotoxic effects of γ-PGA on astrocytes, cells were incubated with increasing concentrations of γ-PGA for 24 h and viability was evaluated using MTT assay. As expected, we observed that γ-PGA had no cytotoxic effect on primary astrocytes at any of the tested concentrations (0.275, 2.75, and 27.5 μM). Surprisingly, we observed a dose-dependent increase in cell viability, with the 27.5 μM concentration significantly improving primary astrocytes viability compared to the control and the 0.275 μM concentration (Fig. 1A). Therefore, 27.5 μM concentration was selected to test the protective or recovery effect of γ-PGA on PFF-treated primary astrocytes, a widely used model to recapitulate the pathological features of astrocytes in PD [23,29]. For this purpose, we used a treatment approach of either adding γ-PGA before (PRE treatment) or after (POST treatment) PFFs administration. As expected, the addition of PFFs to astrocytes decreased cell survival. Interestingly, cell survival was significantly rescued with the POST treatment of γ-PGA (Fig. 1B).

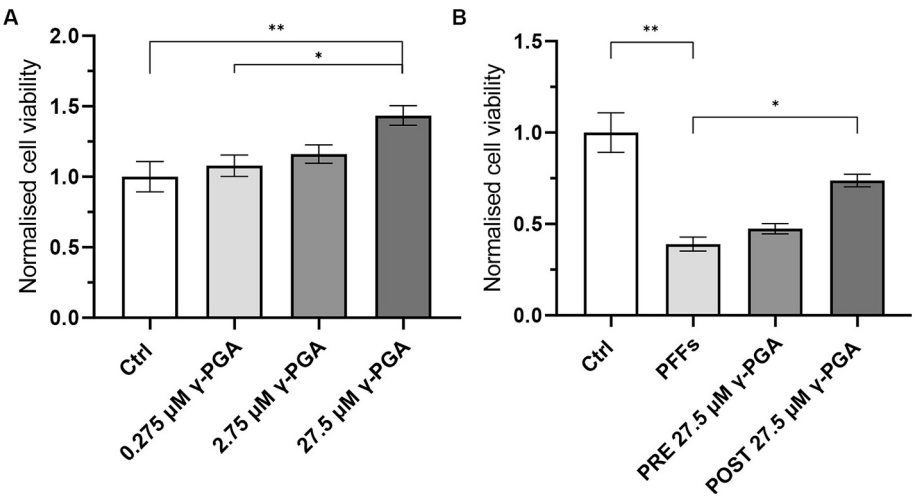


**Fig. 1.** γ-PGA rescues α-synuclein PFF-induced toxicity in primary astrocytes. A) Cell viability of primary astrocytes treated with increasing concentrations of γ-PGA (0.275, 2.75, 27.5 μM). Data are expressed as mean ± SEM, normalized to control and analyzed by ordinary one-way ANOVA with Tukey's *post hoc* test ($n = 5$). B) Cell viability of primary astrocytes treated with α-synuclein PFFs alone and in co-treatment with γ-PGA added before (PRE) or after PFFs (POST). Data are expressed as mean ± SEM, normalized to control and analyzed by Kruskal-Wallis test with Dunn's correction (n = 5), $^{**}p < 0.01$, $^{*}p < 0.05$.

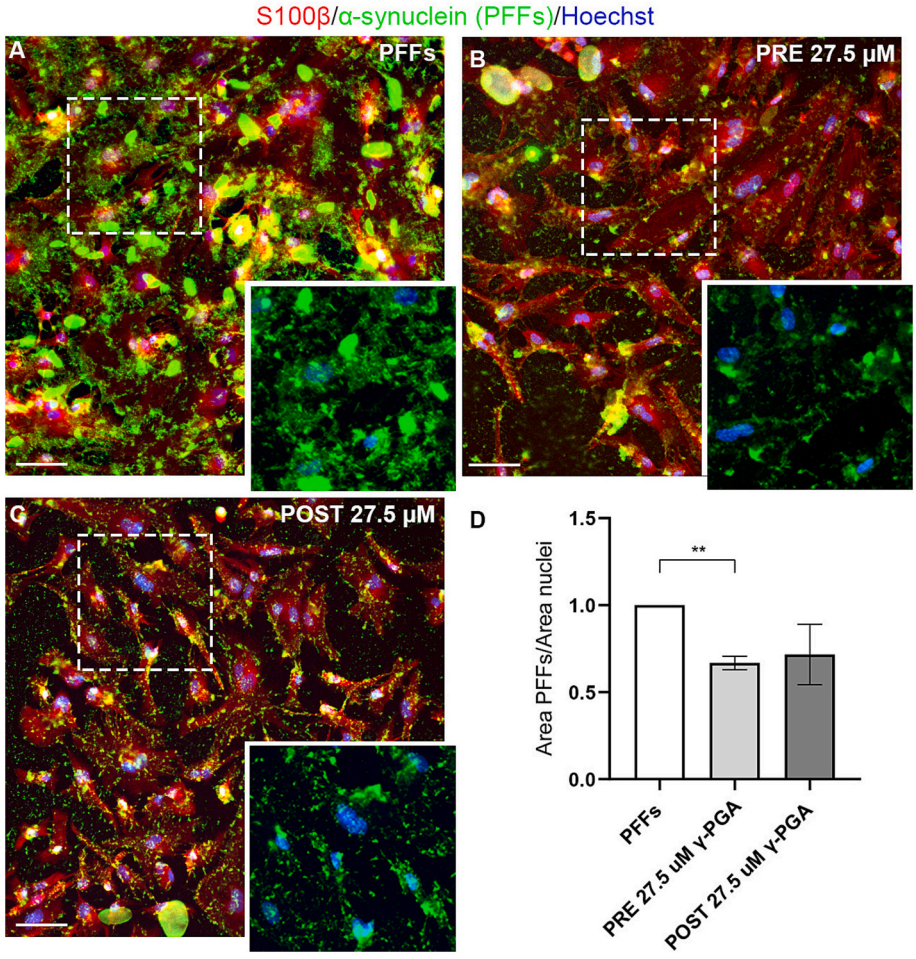


**Fig. 2.** γ-PGA limits the extent of α-synuclein PFFs in primary astrocytes. Spinning disk confocal microscopy images showing α-synuclein PFFs distribution in primary astrocytes, labeled with S100β (A) and treated with γ-PGA before (B) or after (C) the addition of PFFs. Nuclei were counterstained with Hoechst. Scale bar, 50 μm (D). The graph shows the total area of PFFs normalized on the area of nuclei. Data are expressed as fold change of control ± SEM and analyzed by One sample *t*-test ($n = 4$), $^{**}p < 0.01$.

### 3.2. γ-PGA impacts on the distribution of α-synuclein PFFs in primary astrocyte culture

We explored whether the impact of γ-PGA on astrocyte viability could be attributed to its capacity to modulate or interfere with α-synuclein PFFs. Hence, with the same experimental design, we initially analyzed, by immunofluorescence and using an antibody against total α-synuclein, the total area of α-synuclein PFFs (Fig. 2). By adding γ-PGA either before or after PFFs, the extent of α-synuclein aggregates was reduced compared to PFF treatment alone (Fig. 2A, B, C). Notably, the PRE treatment significantly decreased the total area of PFFs compared to PFF treatment alone (Fig. 2D). To further assess how γ-PGA distributes relative to α-synuclein PFFs in astrocytes cell culture, we labeled γ-PGA with FITC and investigated its localization by means of immunofluorescence assay. We observed that when astrocytes were treated with γ-PGA only, γ-PGA distributed mainly inside the cells (Fig. 3A, white arrowheads), as shown by the orthogonal view of the image (Fig. 3A', A"), and by 3D reconstruction with arivis Vision4D® 3.6.0 software (Fig. 3B, B'; white arrowheads; Supplementary Movie 1). When astrocytes were treated with α-synuclein PFFs only, we also observed their intra and extracellular distribution (Fig. 3B, white arrowheads and arrow, respectively). Z-axis orthogonal projections of confocal multiplane images revealed the internalization of α-synuclein PFFs within astrocytes (Fig. 3C-C"), further confirmed by the 3D reconstruction with arivis Vision4D® 3.6.0 software (Fig. 3D', white arrowheads; Supplementary Video 2). Lastly, when γ-PGA was co-administered with

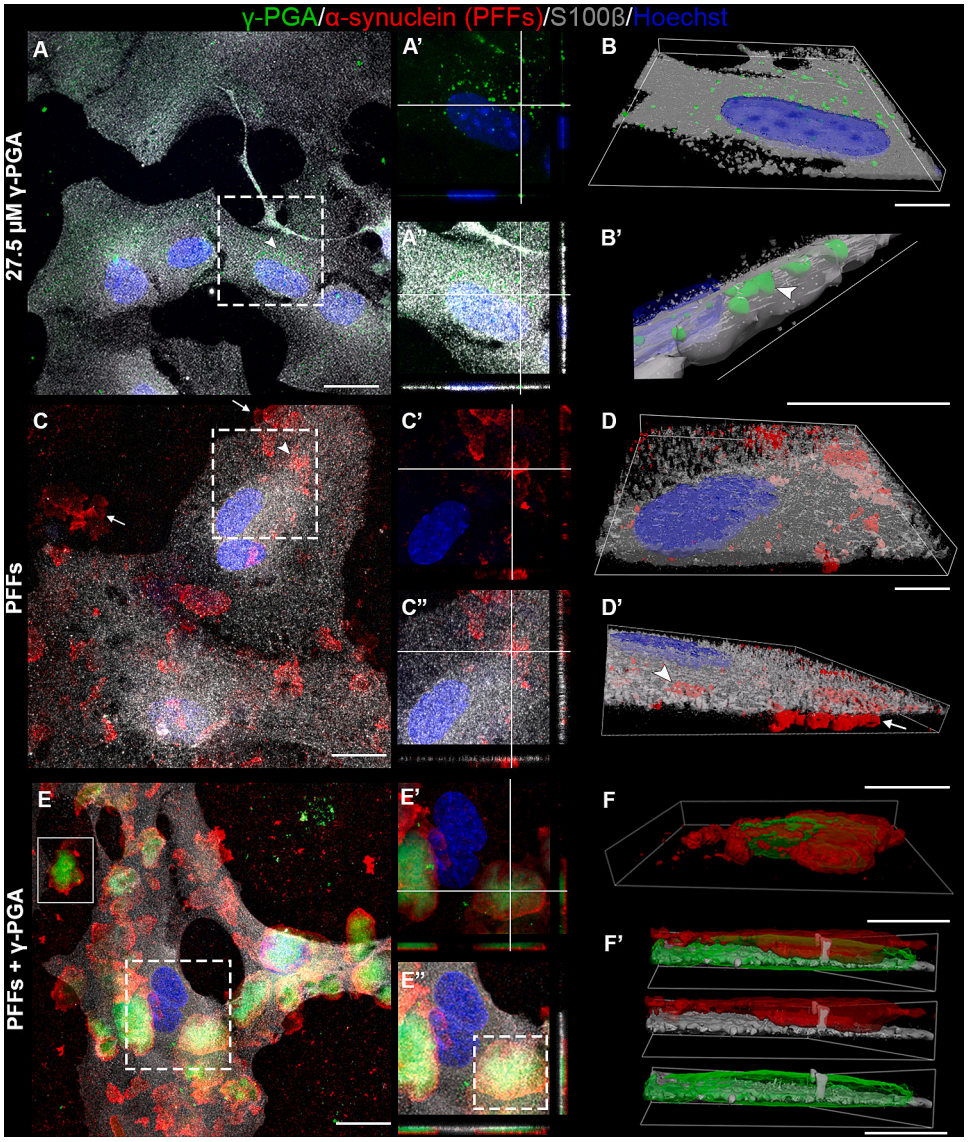


**Fig. 3.** The interplay between γ-PGA and α-synuclein PFFs in primary astrocytes. **A)** Spinning disk confocal images showing the distribution of γ-PGA in murine primary astrocytes. A' and A" show the magnification of the dashed rectangle in A and the orthogonal projections that represent the XZ (bottom) and YZ (right) planes and highlight the presence of γ-PGA inside the cell. B and B′ show the top and side view of the 3D reconstructions obtained with arivis Vision4D® 3.6.0 software of A". C) Spinning disk confocal images showing α-synuclein PFFs distribution in astrocytes. C′ and C″ show the magnification of the dashed rectangle in C and the *Z*-axis projection. D and D′ show the top and side view of the 3D reconstructions obtained with arivis Vision4D® 3.6.0 software of C. E) Representative spinning disk confocal image of PRE treatment with γ-PGA of primary astrocytes. E' and E" show the magnification of the dashed rectangle in E and orthogonal view showing the distribution of γ-PGA relative to α-synuclein PFFs. F represents the 3D reconstruction by arivis Vision4D® 3.6.0 software of the rectangle in E showing γ-PGA and α-synuclein PFF colocalization in the extracellular environment. F′ illustrates the lateral view of 3D reconstruction of the dashed rectangle in E"; the split channels highlight the disposition of γ-PGA and α-synuclein PFFs when compared to S100β labeled astrocytes. Nuclei were counterstained with Hoechst. White arrows and white arrowheads in A, B′, C and D′ show the intracellular and extracellular localization, respectively, of either y-PGA or PFFs. A, A', A", C, C′, C″, E, E', E": scale bar, 20 µm. B, B′, D, D′, F, F′: scale bar, 5 µm.

α-synuclein PFFs to primary astrocytes, we observed that γ-PGA colocalized with α-synuclein PFFs in both PRE treatment (Fig. 3E, F, Supplementary Movie 3) and POST treatment (data not shown). Moreover, upon observing their arrangement relative to the cell, γ-PGA clearly localized between α-synuclein PFFs and S100β-labeled astrocytes, as shown in the orthogonal projection (Fig. 3E, E") and 3D reconstruction (Fig. 3F').

### 3.3. γ-PGA limits the internalization of α-synuclein PFFs by primary astrocytes

The amount of α-synuclein PFFs within astrocytes is a key factor, which affects their ability to clear it, whereby excessive accumulation could impair the protein's degradation pathway [30]. Thus, we explored whether γ-PGA could have any impact on the internalization of PFFs by astrocytes. To evaluate this hypothesis, we labeled α-synuclein PFFs with a pH-sensitive dye (pHrodo-PFFs) that becomes fluorescent when the pH of the surrounding environment increases in acidity, such as late endosomes and lysosomes. We noted that fluorescence area and intensity of pHrodo-PFFs were significantly lower following both γ-PGA treatments (Fig. 4B, C) compared to pHrodo-PFFs alone (Fig. 4A). Notably, γ-PGA PRE-treatment was the most effective in reducing α-synuclein PFF internalization (Fig. 4D). In parallel, we explored whether γ-PGA may affect the degradation of PFFs by analyzing the autophagic and lysosomal pathways involved in their clearance [31,32]. To assess the effect of γ-PGA treatment on autophagy, we examined LC3 and p62 levels by Western blotting. Treatment with α-synuclein PFFs led to a significant increase of LC3-II/LC3-I ratio (Fig. 4E, F) and accumulation of p62 (Fig. 4E, G), suggesting an impairment in the autophagic flux. Although no differences were observed with PRE-treatment of γ-PGA, POST treatment resulted in a mild decrease in p62 levels (Fig. 4E, G). With respect to the lysosomal pathway, we employed LAMP1 as a marker of autophagy-related lysosomal organelles [33], and found an increase in LAMP1 fluorescence area and intensity with PFF treatment, that was not altered by γ-PGA treatment (Fig. S2). These results suggest that γ-PGA mainly exerts its effect by limiting PFF internalization in primary astrocytes.

### 3.4. γ-PGA prevents and recovers PFF-induced inflammation in primary astrocytes

It is widely recognized that astrocytes play a key role in the inflammatory response during PD pathogenesis [17]. Several studies have shown that α-synuclein aggregates can activate the astrocytic inflammatory response inducing the acquisition of a reactive phenotype that exacerbates the pathological condition [29,34]. Thus, given the anti-inflammatory properties of γ-PGA [4,5], we tested whether this biomacromolecule could prevent or alleviate astrocytes inflammation induced by α-synuclein PFFs. Immunofluorescence assay was employed to evaluate Complement C3 protein, a marker of A1 reactive astrocytes [21] (Fig. 5). As expected, γ-PGA alone had no effect on C3 protein in terms of both fluorescence area and intensity (Fig. S3) whereas treatment with α-synuclein PFFs increased C3 deposition compared to the control (Fig. 5A, B). Both PRE and POST treatment with γ-PGA significantly reduced fluorescence area and intensity of C3 staining compared to PFFs (Fig. 5C, D, E, F). Notably, we did not observe any significant difference in γ-PGA anti-inflammatory properties between PRE and POST treatment (Fig. 5E, F).

To further investigate the inflammatory response, we measured the levels of pro-inflammatory cytokines released by treated astrocytes, including TNF-α, IL-1β, IL-6, and the chemokine CXCL10, which have previously been reported to be elevated in PFF treated astrocytes [24,35]. In both control and γ-PGA-treated astrocytes, we detected very low levels of CXCL10 and IL-1β release (Fig. 5G, H), while no detectable levels of TNF-α and IL-6 were observed (Fig. 5I, J). As expected, PFF treatment led to a significant increase in the release of CXCL10, IL-1β and TNF-α, which was significantly attenuated following POST treatment with γ-PGA (Fig. 5G, H, I). Moreover, no differences were found between control and POST treatment. Regarding IL-6, γ-PGA POST treatment showed a trend toward reduced release in PFF treated cells (Fig. 5J). Collectively, these findings support the role of γ-PGA in attenuating the inflammatory response induced by α-synuclein PFFs.

### 3.5. γ-PGA affects α-synuclein aggregation in vitro

To thoroughly investigate whether γ-PGA interferes with α-synuclein aggregation *per se*, we performed *in vitro* studies and showed the biopolymer's capacity to delay the aggregation of α-synuclein in a dose-dependent manner (Fig. 6A). In particular, the green curve shows the aggregation kinetics of α-synuclein in the absence of γ-PGA. The pink, blue and red curves refer to the experimental conditions with increasing concentrations of γ-PGA at 2.75 μM, 27.5 μM and 275 μM, respectively. Remarkably, the 27.5 μM and the 275 μM concentrations were the most effective in inhibiting the aggregation of α-synuclein. Notably, at the concentration of 275 μM, aggregation did not occur even after 40 h. The gray curves in the graph refer to γ-PGA alone (at the different concentrations tested: 2.75 μM, 27.5 μM, and 275 μM) and none of them show any propensity to undergo self-assembly.

Samples collected following incubation with 27.5 μM of γ-PGA, were analyzed by electron microscopy to better characterize the effect of the polymer on the presence and the structure of α-synuclein aggregates. Following γ-PGA incubation with monomeric α-synuclein, no fibrils were observed, while oligomer-like structures were detected (Fig. S4). This strengthens the role of γ-PGA in interfering with α-synuclein aggregation.

The highest concentration of γ-PGA (275 μM) was used to treat PFFs before testing their ability to trigger the aggregation of α-synuclein (Fig. 6B). Both treated and untreated PFFs (purple and orange curves, respectively) were able to accelerate the aggregation kinetics of α-synuclein (green curve). Interestingly, a trend toward attenuation of PFF effect is observed when preincubated with γ-PGA.

## 4. Discussion

γ-PGA is an innovative, non-toxic, non-immunogenic biopolymer whose biosynthetic pathway allows the producer to create γ-peptidic bonds with variations in both molar mass and crystallinity [2]. This unique feature has led to growing interest in γ-PGA, owing to its diverse properties that make it suitable for a variety of research areas, including biomedicine [36,37]. Among its features, the antioxidant, anti-inflammatory, and neuronal cell death-attenuation properties are remarkable [3–6]. This study explored, for the first time, the biological effect of γ-PGA in the field of PD. γ-PGA can be synthesized naturally by *Bacillus subtilis* during the fermentation of Natto beans. These traditional soybean fermented foods are highly consumed in Japan where, interestingly, the relative PD incidence rate is reported to be significantly lower than in Europe [38]. Given the properties of γ-PGA, we investigated its biological effect on murine primary astrocytes treated with α-synuclein PFFs as a model of PD for the study of cytotoxicity, neuroinflammation, and protein clearance. Indeed, astrocytes play a crucial role in PD pathogenesis by regulating both the clearance of extracellular aggregated α-synuclein and the modulation of neuroinflammatory responses [20,39]. We chose two types of treatment by either administering γ-PGA before or after α-synuclein PFFs to explore its protective or rescue effect on murine primary astrocytes. The major finding of our study is that γ-PGA reverses the cytotoxic effects of α-synuclein PFFs, acts on the ability of astrocytes to internalize them and decreases cellular inflammation, as summarized by the proposed model in Fig. 7.

Given that one of the main challenges in the neurodegeneration field is to design protective strategies based on chemical or biological drugs, our work aims to tackle this by exploring the properties of γ-PGA.

Emerging research has demonstrated the non-toxic and excellent

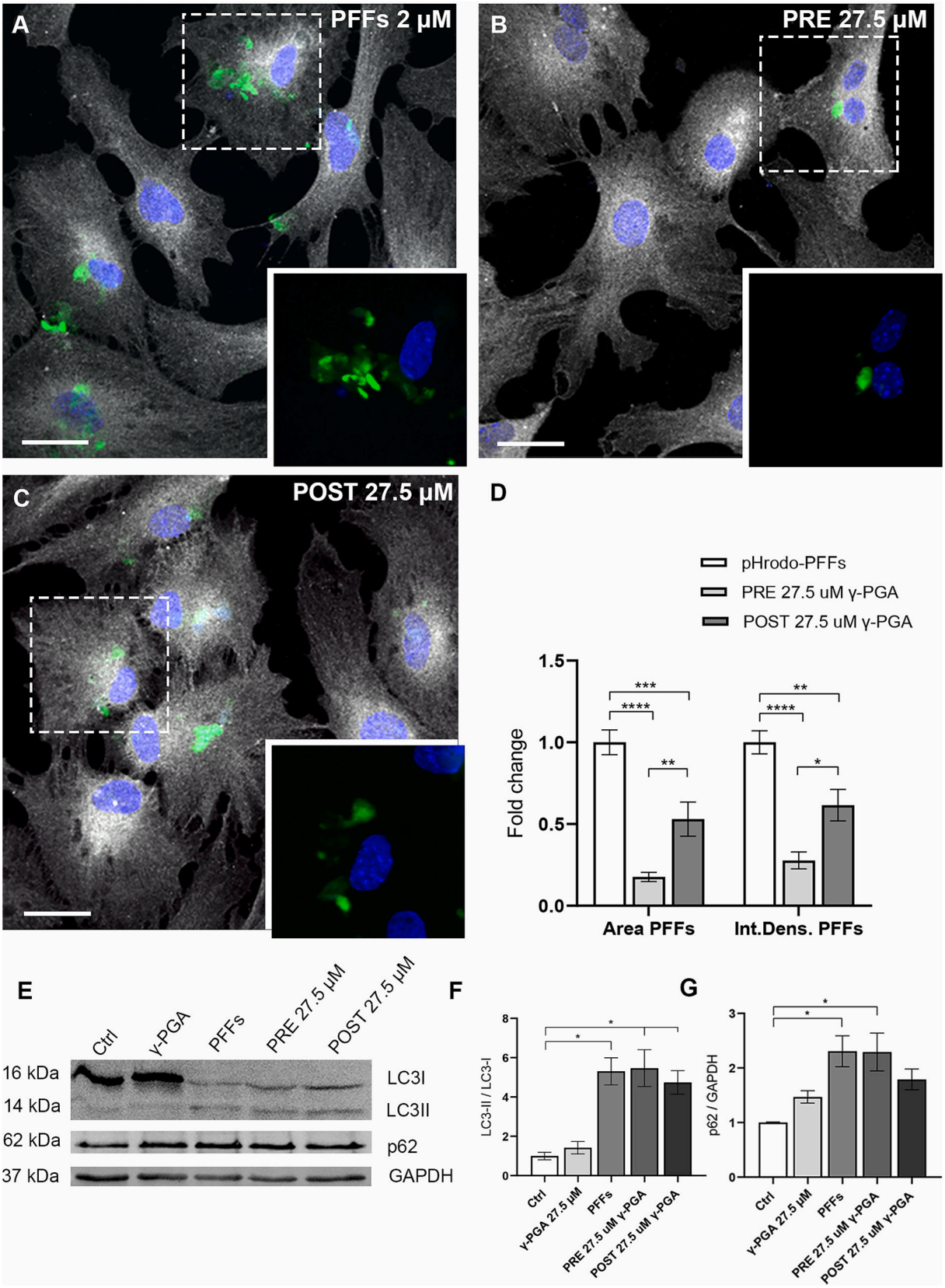


**Fig. 4.** γ-PGA administration decreases PFFs internalization by primary astrocytes. Maximum intensity Z-projection confocal images of primary astrocytes labeled with S100β and treated with pHrodo-PFFs (A) and with γ-PGA added before (B) or after (C) PFFs. Nuclei were counterstained using Hoechst. Scale bar, 20 μm. D) The graph shows the area covered by pHrodo-PFFs and pHrodo-PFF fluorescence intensity (Integrated Density) both normalized on the area of nuclei. E) Representative immunoblots for LC3 (LC3I, 16 kDa; LC3II, 14 kDa), p62 (62 kDa) and GAPDH (37 kDa). G) and H) graphs show LC3-II/LC3-I ratio and quantification of p62 normalized over GAPDH. Data are reported as mean ± SEM, normalized to control and analyzed by ordinary one-way ANOVA with Tukey's *post hoc* test ($n = 4$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

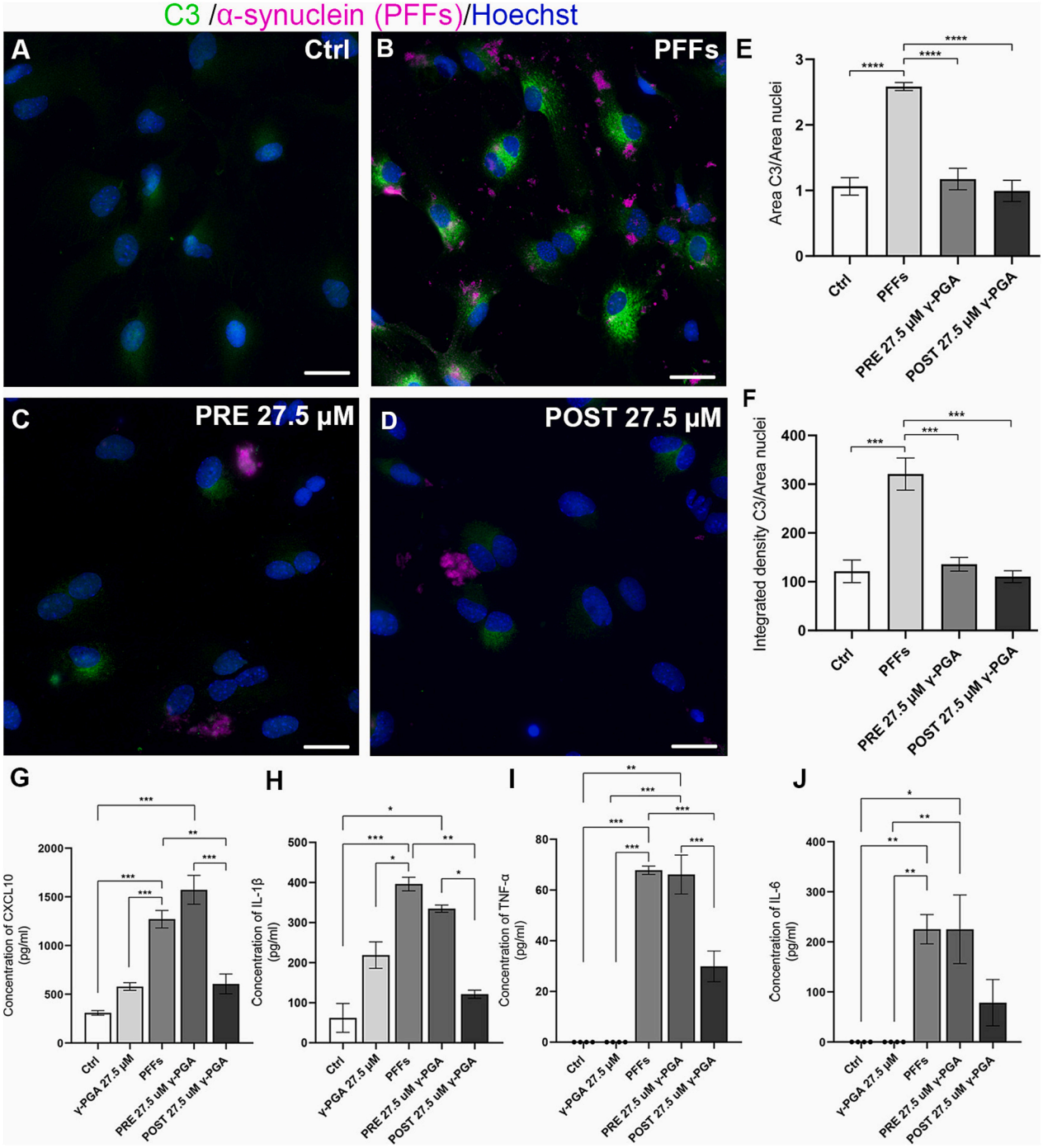


**Fig. 5.** γ-PGA administration decreases PFF-induced inflammation in primary astrocytes. Representative confocal images showing complement C3 staining in primary astrocytes in control condition (A), treated with PFFs (B) and with γ-PGA added before (C) or after (D) the addition of PFFs. Nuclei were counterstained using Hoechst. Scale bar, 20 μm. The Graphs show area (E) and fluorescence intensity (Integrated density) (F) of C3 protein normalized on the area of nuclei. Graphs G, H, I and J respectively show CXCL10, IL-1β, TNF-α, and IL-6 release by primary astrocytes alone or in the presence of γ-PGA, PFFs or the combination of the two, quantified by ELISA. Data are reported as mean ± SEM and analyzed by ordinary one-way ANOVA with Tukey's *post hoc* test (n = 4), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

safety profile of γ-PGA in diverse experimental settings [2,6]. Here, we tested whether γ-PGA could affect primary murine astrocyte survival and reported that, it increased cell viability in a dose-dependent manner. In the context of α-synuclein PFFs, multiple evidence suggests that aggregated α-synuclein species induce toxicity [40]. However, the mechanisms are still debated. Some studies hypothesize that the interaction of aggregated α-synuclein with cellular membranes induces their disruption, activation of inflammatory pathways, autophagic and lysosomal processes, and cell death through apoptosis [24,41]. We report that α-synuclein PFFs decrease astrocyte viability and that γ-PGA, can counteract PFF-induced toxicity, suggesting that the polymer may have cell-protective effects. Our results could be explained in two possible ways. First, cell protection could be due to the potent antioxidant properties of γ-PGA that can act as ion chelator, free radical scavenger, and inhibitor of lipid peroxidation [6]. Indeed, we demonstrated, for the first time, that γ-PGA is readily internalized by astrocytes, with

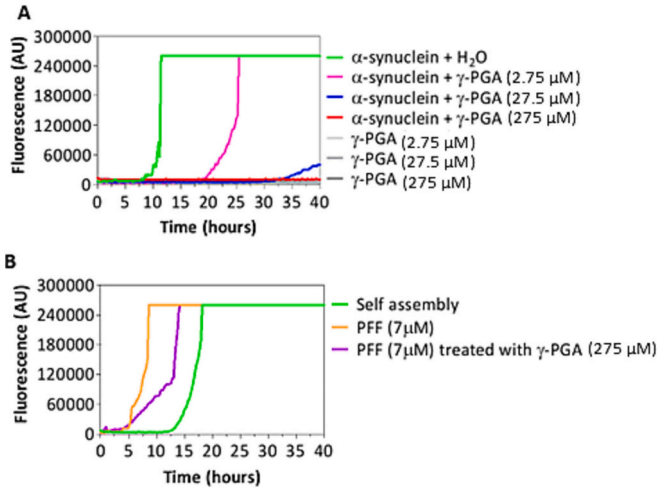


**Fig. 6.** γ-PGA affects α-synuclein aggregate formation *in vitro*. A) Aggregation kinetics of monomeric α-synuclein supplemented with different concentrations of γ-PGA: 2.75 μM (pink curve), 27.5 μM (blue curve) and 275 μM (red curve). The gray lines refer to γ-PGA alone (2.75, 27.5, 275 μM). B) Aggregation kinetics of monomeric α-synuclein in the presence of PFFs either treated with 275 μM of γ-PGA (purple curve) or untreated (orange curve). The graphs show the average fluorescence intensity of the four replicates for each curve plotted against time.

diffuse, punctate cytoplasmatic localization. Secondly, γ-PGA may exert a direct buffering effect on α-synuclein PFFs thereby reducing their toxicity.

Looking at the interplay between γ-PGA and α-synuclein, our data gathered from *in vitro* α-synuclein aggregation assay suggest that γ-PGA could interfere with the aggregation process in a dose-dependent manner, with a trend toward attenuation of PFF assembly even when incubated with pre-formed aggregates. Moreover, incubation with γ-PGA seems to reduce the presence of fibrils, along with the appearance of small α-synuclein oligomer-like structure, suggesting that γ-PGA may influence α-synuclein aggregation in a cell-free system. Nevertheless, the polydispersity of α-synuclein PFFs should be considered when interpreting this type of *in vitro* assays, as the impact of γ-PGA could only involve certain subtypes of fibrils, size or morphology dependent. Further analyses in this direction may be needed to unveil the exact nature of the interaction.

In addition, we showed that the two proteins colocalize in both extracellular and intracellular environments, and that both POST and PRE treatments with γ-PGA are effective in reducing α-synuclein PFF burden. This might suggest that γ-PGA may, at least to some extent, disrupt α-synuclein aggregates or, alternatively, reduce their interaction with astrocytes, potentially affecting their internalization. Indeed, the internalization of extracellular α-synuclein aggregates by astrocytes is a key aspect in neurodegenerative processes, as astrocytes are known to be actively involved in their clearance [20,26,42]. Nevertheless, the amount of α-synuclein uptake is critical for astrocyte homeostasis as excessive accumulation could overwhelm protein degradation processes leading to cellular stress [20,42]. Several mechanisms may be involved in the entry of PFFs into the cells, such as direct membrane penetration [43], caveolae-mediated [44] and clathrin-dependent endocytosis [45]. In addition, one study showed that PFF uptake by astrocytes could be mediated by dynamin-mediated endocytosis as dynamin facilitates the budding of endocytic vesicles from the plasma membrane [26].

Here, we found that γ-PGA has a marked inhibitory effect on astrocyte ability to internalize α-synuclein PFFs (Fig. 7B, a). Interestingly, the PRE treatment reduced both the size and the intensity of internalized α-synuclein PFFs to a greater extent, compared to POST treatment. In respect to the mechanisms by which γ-PGA limits α-synuclein PFF internalization, we propose two hypotheses. Firstly, γ-PGA could compete for cellular uptake pathways employed by α-synuclein aggregates. This hypothesis is supported by our findings on both the presence of γ-PGA within astrocytes (Fig. 7B, b), and its localization between the cell and α-synuclein PFFs (Fig. 7B, c). The second hypothesis builds upon our results on the colocalization of the two molecules, suggesting that γ-PGA may have a buffering effect and retain PFFs, preventing their internalization (Fig. 7B, d). Moreover, we investigated the lysosomal and autophagic pathway which is responsible for clearing cytosolic aggregated proteins such as fibrillar α-synuclein [31]. We observed that treatment with PFFs impairs autophagy-lysosome pathways, as suggested by the increase of LC3-II/LC3-I ratio, the accumulation of p62, and the increase of LAMP1-labeled lysosomes, as previously demonstrated [31,32]. Interestingly, POST treatment with γ-PGA led to a mild decrease of p62 accumulation, which might suggest a partial role in modulating autophagy. However, our results support the hypothesis that γ-PGA primarily exerts its effect by limiting PFF internalization in astrocytes. Despite we also observed a reduction in the overall extent of extracellular aggregates, further studies are required to better

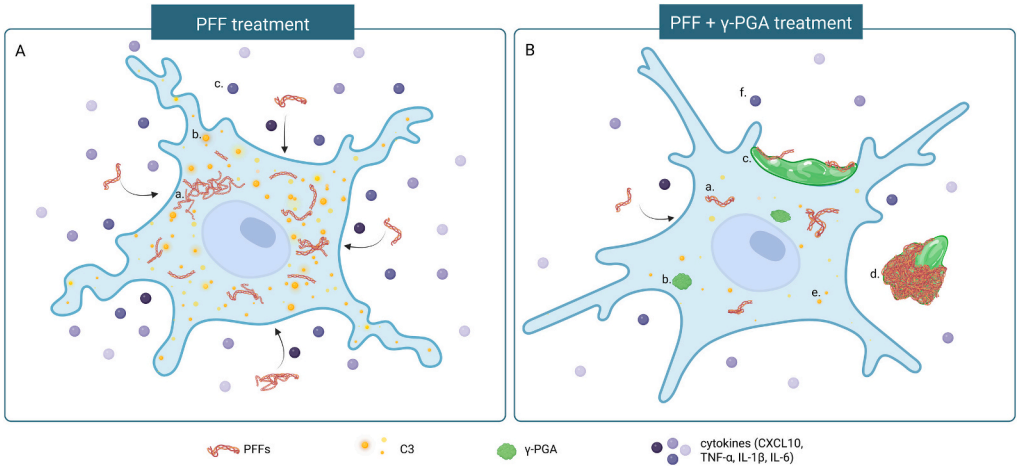


**Fig. 7.** Schematic illustration of γ-PGA effect on PFF-treated primary astrocytes. A) In primary astrocytes that are treated with PFFs, the fibrils are internalized by the cell (a), and an inflammatory response is observed, as shown by C3 staining (b), and by the release of pro-inflammatory cytokines (c); this results in cell toxicity. B) In primary astrocytes co-treated with γ-PGA, a reduction in PFF internalization is observed (a). γ-PGA localizes inside the cell (b), on its surface (c), and colocalizes with PFFs in the extracellular space (d). A decrease in C3 inflammation and pro-inflammatory cytokines release is also observed (e, f), leading to reduced cellular toxicity. Created in BioRender. Cappelletti, G. (2025) https://BioRender.com/bbanupg.

investigate the buffering capacity of γ-PGA in limiting α-synuclein spreading and its effect on neurons.

A relevant feature of astrocytes is that they are active players in neuroinflammation, and their response may be beneficial or detrimental, depending on the kind of stimuli they encounter [39]. Previous studies have demonstrated that extracellular α-synuclein aggregates, such as α-synuclein fibrils, induce innate immune pathway activation such as Toll-like receptor 4-mediated pathways, causing inflammatory responses in astrocytes [46]. However, although it is still debated whether adult astrocytes express TLR4 *in vivo* [21,47,48], it has been stated that innate Complement component 3 (C3) is one of the most distinctive and highly upregulated genes in neurotoxic and pro-inflammatory astrocytes, namely A1 astrocytes [21,49]. Indeed, *in situ* hybridization and qPCR assays for C3 on *post-mortem* human brain of PD patients have revealed that $C3^{+}$ astrocytes abundance is significantly increased compared to controls [21]. Therefore, we investigated γ~~-PGA~~ the effect of γ-PGA on α-synuclein PFF-induced inflammation of astrocytes by evaluating C3 as an inflammatory marker. Our results suggest, in accordance with what has been previously shown [50], that α-synuclein PFFs increases C3 deposition in astrocytes (Fig. 7A, b) and that γ-PGA can both prevent and recover astrocytes inflammation (Fig. 7B, e). Given that C3 expression in α-synuclein PFF-treated astrocytes has been reported to induce neuronal cell death [50], a molecule capable of mitigating complement activation in astrocytes holds significant promise as a therapeutic strategy for PD. Moreover, we observed a pro-inflammatory profile in α-synuclein PFF-treated astrocytes, characterized by increased release of TNF-α, IL-1β, IL-6 and CXCL10 (Fig. 7, c). High levels of TNF-α, IL-1β and IL-6 can promote glial reactivity, contribute to chronic inflammation and neuronal dysfunction, while CXCL10 can lead to sustained neuroinflammation and immune cell infiltration, ultimately disrupting neural homeostasis [51]. In our study, we found that POST treatment with γ-PGA led to a mild decrease in IL-6 release, while significantly reduced TNF-α, IL-1β and CXCL10 levels in astrocytes treated with PFFs (Fig. 7, f). Thus, even though the PRE treatment may provide a greater reduction in α-synuclein PFF internalization, our results on the autophagic pathway and on astrocyte inflammation indicate POST treatment as the most promising therapeutic strategy.

## 5. Conclusions

Overall, these data provide a "proof of concept" that bacterial-derived γ-PGA may be considered a promising candidate for mitigating α-synuclein pathology. Adding γ-PGA after PFF exposure seems to be more effective than preventive treatment and this is essential for any therapeutic approach targeting PD, where neuroinflammation is already ongoing and α-synuclein aggregates already exist and need to be effectively 'inactivated' or removed.

Nevertheless, many aspects remain to be elucidated including the molecular mechanisms underlying its biological effect shown here, its ability to interfere with other aggregate species, including α-synuclein oligomers, and its potential beneficial effect on neurons. Interestingly, γ-PGA is a well-characterized prebiotic able to increase the species associated with a healthy microbiome; this feature is promising given the microbiota dysbiosis, the high level of inflammation, and the presence of α-synuclein aggregates described in the gut of PD patients.

We supplied a file as Supporting information that includes complementary data (PDF). This file includes the characterization of α-synuclein PFFs, LAMP1 quantification, the effect of γ-PGA on astrocyte inflammation (C3 staining), and electron microscopy analyses of α-synuclein and γ-PGA after *in vitro* aggregation assay. Next, we supplied 3 videos (.mp4): Supplementary Video 1 (the movie refers to the 3D reconstructions obtained with arivis Vision4D® 3.6.0 software of Fig. 3B); Supplementary Video 2 (the movie refers to the 3D reconstructions obtained with arivis Vision4D® 3.6.0 software of Fig. 3D); Supplementary Video 3 (the movie refers to the 3D reconstructions obtained with arivis Vision4D® 3.6.0 software of Fig. 3E). Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.145303.

## CRediT authorship contribution statement

**Claudia Novello:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Mattia Parati:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Samanta Mazzetti:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Oriana Rampoldi:** Investigation, Formal analysis. **Huseyin Berkcan Isilgan:** Investigation, Formal analysis. **Milo Jarno Basellini:** Investigation, Formal analysis. **Chiara M.G. De Luca:** Investigation, Formal analysis. **Arianna Ciullini:** Investigation, Formal analysis. **Ilaria L. Dellarole:** Investigation, Formal analysis. **Alessandro Fantin:** Investigation, Formal analysis. **Isabella Russo:** Writing – review & editing. **Brian L. Johnston:** Writing – review & editing. **Moira Paroni:** Formal analysis, Writing – original draft, Writing – review & editing. **Chiara Rolando:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Fabio Moda:** Writing – review & editing, Investigation, Formal analysis. **Gianni Pezzoli:** Writing – review & editing. **Iza K. Radecka:** Writing – review & editing. **Graziella Cappelletti:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization.

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## Declaration of competing interest

The authors declare no competing financial interest.

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## Data availability

The authors confirm that the data supporting the findings of this study are available within the article and Supplementary materials.

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