Formulation development of tocopherol polyethylene glycol nanoengineered polyamidoamine dendrimer for neuroprotection and treatment of Alzheimer disease

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ABSTRACT
Amyloid-beta (Aβ) aggregates deposition at extra neuronal sites induces neurotoxicity and major hallmarks of Alzheimer’s disease (AD). To reduce the Aβ fibril toxicity, multi-functional polyamidoamine (PAMAM) dendrimer was conjugated with tocopherol polyethylene glycol succinate-1000 (TPGS) which acts as a carrier matrix for the delivery of neuroprotective molecule piperine (PIP). This PIP-TPGS-PAMAM dendrimer was fabricated to mitigate the Aβ fibril toxicity on SHSY5Y cells. TPGS-PAMAM was fabricated through carbodiimide coupling reaction, and PIP was encapsulated in dendrimer through solvent injection method to prepare PIP-TPGS-PAMAM. Antioxidant assay of PIP-TPGS-PAMAM showed 90.18% inhibition of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals compared to free PIP, which was 28.27%. The SHSY5Y cells showed 37.25% for negative control group and 82.55% cell viability for PIP-TPGS-PAMAM treated group. In addition, PIP-TPGS-PAMAM also disaggregated the Aβ fibril in SHSY5Y cells. Our findings suggested that PIP-TPGS-PAMAM showed mitigation of Aβ fibril toxicity in neuronal cells, which can offer excellent prospect of neuroprotection and AD therapy.

Introduction
Alzheimer’s disease (AD) is associated with degeneration of neuronal cells, which lead to cognitive decline brain. AD majorly affects hippocampus and cerebral cortex of brain due to aggregates of amyloid beta (Aβ) protein [1]. Aβ monomers oligomerise to form aggregates via self-recognition binding of monomers of protein. In AD, production of the Aβ in brain higher compared to the clearance rate results in deposition of aggregates at extra neuronal sites [2]. The extracellular deposition of Aβ aggregates triggers formation of reactive oxygen species (ROS), which leads induction of apoptosis in neuronal cells [3]. Current treatment interventions like acetylcholine esterase inhibitor and NMDA (N-methyl D-aspartate) antagonist gives only symptomatic relief in AD [4]. Therefore, inhibition of Aβ aggregate formation and scavenging of ROS can be alternative therapeutic method to reduce neurotoxicity in AD models.

Piper longum active constituent piperine (PIP) is known for its profound effect on the central nervous system (CNS) has been reported to improve cognitive function in AD. PIP is a nitrogenous pungent alkaloid compound that is extracted from the fruit of Piper longum having an aqueous solubility of 40 mg/L. Various reported experimental results have shown that PIP can increase cognitive function, the structural activity relationship (SAR) of PIP due to a polyene double bond system in chemical structure that acts as potent antioxidant. The tertiary nitrogen involved in PIP moiety mimics acetylcholine esterase’s inhibitor drug in brain [5,6]. Reportedly, PIP demonstrated protective effect on neuronal SHSY5Y cells against Aβ mediated cytotoxicity by scavenging the ROS generation [7]. Although with aforementioned advantages, CNS delivery of PIP is challenging due to its poor water solubility which restricts entry in neuronal cells [8]. Nanomedicines have wide application in encapsulation of hydrophobic drugs and increase their availability to targeted site. Nanocarriers comprising of polymers, lipids and inorganic materials are developed, which demonstrated excellent properties for targeting CNS with neuroprotective agents [9]. Among all nanocarriers, dendrimers exhibited desired properties of drug delivery systems such to increased solubility, improved half-life, greater permeation of drugs, ability to encapsulate hydrophobic and hydrophilic molecules, site-specific targeting and superficial passage across cell membrane by transcytosis [10–14]. Polyamidoamine (PAMAM) dendrimers of G4 generation are highly branched and monodisperse macromolecule consists of free amine groups. These free amines facilitates
covalent attachment of ligands, targeting agents and drugs. The hydrophobic core of PAMAM is advantageous for physical encapsulation of lipophilic drugs [15-24]. PAMAM dendrimer has ability to inhibit the Aβ fibril formation by attaching to free end of peptide and terminates fibril growth thermodynamically. These dendrimers exhibit generation dependent disaggregation effect on the Aβ fibrils; in addition, PAMAM can also reduce the preformed Aβ fibrils [25,26]. However, toxicity associated with PAMAM has been the major concern due to positively charged free amine groups during intracellular drug delivery [27]. In another study, grafted PAMAM dendrimer has shown the Aβ disaggregation through nucleophile reaction with lysine terminal end of Aβ protein [28], whereas hydroxyl modified PAMAM dendrimer are non-cytotoxic and disaggregate Aβ fibrils of human islets. The multiple hydroxyl group on the surface of dendrimers has shown their ability to interact with Aβ with no significant cell death. Grafting the surface of PAMAM dendrimer enhances cellular biocompatibility and showed interesting anti-amyloidogenic properties [29].

To overcome the toxicity associated with free amine without hampering the amyloid plaque disaggregation activity, the PAMAM dendrimer surface was modified with ω-D-Tocopheryl Polyethylene Glycol Succininate 1000 (TPGS). TPGS is amphiphilic biocompatible macromolecule polymer that includes polar head and lipophilic tocopherol tail. Tocopherol imparts antioxidant property in TPGS and functions as free radical scavenger of ROS. It enhances the drug permeation through lipophilic cell membranes and improves the availability of drugs at intra-neuronal sites. In addition, tocopherol prevents amyloid-induced neurotoxicity including oxidative stress and lipid peroxidation in SH-SYSY cells [30].

In this work, PAMAM G4 generation dendrimer is conjugated with TPGS to form TPGS-PAMAM in two steps reactions via esterification and amide coupling. This TPGS-PAMAM was used to encapsulate PIP molecules through solvent injection method to form PIP-TPGS-PAMAM. This nanocarrier was screened for neuroprotective and Aβ plaque disaggregation activity. The Aβ disaggregation activity was monitored by various spectral, chemical and microscopy-based techniques. Further neuroprotection activity of PIP-TPGS-PAMAM was evaluated on Aβ-induced cytotoxicity on SH-SYSY cell line and assessed for cell viability assay, ROS scavenging assay, apoptosis and amyloid aggregated detection using fluorescent microscopy.

Materials

PAMAM dendrimer G4.0 generation, TPGS, dimethylaminopropyridine (DMAP), piperine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl), 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent, Aβ1-42 rat, 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA), dianiminophenyl indole (DAPI), Thioflavin T (ThT) and Annexin-FITC apoptosis kit were purchased from Sigma-Aldrich (USA), Succinic anhydride (SA), Trinitrobenzenesulphonic acid (TNBS) reagent, Dulbecco modified Eagles medium (DMEM) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylylazotetrazolium bromide (MTT) was obtained from Himedia. Triple distilled water (TDW) was obtained from Milli-Q system (Millipore, Merck). Solvents including methanol, dimethyl sulphoxide, acetone, hexafluoropropiovanol (HFIP) and dichloromethane (DCM) were purchased from MERCK INDIA.

Methods

Synthesis

Succinylation of TPGS to TPGS-COOH

The carboxylic group introduced in TPGS molecule through esterification reaction by adding succinic anhydride (SA), briefly TPGS (500 mg, 0.869 mmol), SA (173 mg, 1.73 mmol) and DMAP (122 mg, 0.869 mmol) were co-dissolved in 10 mL anhydrous DCM. The reaction mixture was stirred for 24 h in an inert atmosphere at room temperature. The product was suspended in cold DCM to filter out the unreacted SA. Then TPGS-COOH precipitated in 10 mL of diethyl ether and vacuum dried [31].

Conjugation of PAMAM with TPGS-COOH (TPGS-PAMAM)

PAMAM G4 dendrimer (25 mg, 1.76 mmol) was dispersed in 5 mL of triple distilled water (TDW). Then 50 molar equivalents TPGS-COOH (39 mg, 5.98 mmol) was activated adding EDC (11.4 mg, 5.9 mmol) with magnetic stirring for 4 h. Activated TPGS-COOH was added dropwise to PAMAM dendrimer aqueous solution with high-speed stirring and reaction mixture was kept for 24 h at room temperature. Then reaction mixture was purified using dialysis-sibsis membrane of molecular weight (MW) cut off 12000 Daltons (Da). To remove free reactants, phosphate buffer saline (PBS) medium was used for dialysis to obtain TPGS-PAMAM. For drying, TPGS-PAMAM was lyophilised using freeze dryer (LAB CONCO).

Preparation of PIP encapsulated TPGS-PAMAM dendrimer (PIP-TPGS-PAMAM)

Briefly weighed 5 mg of PIP was co-dissolved with 50 mg of TPGS-PAMAM dendrimer in 2 mL of methanol. This organic phase was added in an aqueous solution dropwise through syringe. The mixture is stirred at 700 rpm on magnetic stirrer overnight at ambient temperature to evaporate the methanol from solution. Excess saturated PIP was removed from solution via filtration through 0.4μm syringe filter to obtain PIP-TPGS-PAMAM nanosuspension. Similarly, non-conjugated PIP-PAMAM were also prepared for further studies.

Characterisation techniques

1H NMR

The NMR spectra of TPGS, TPGS-COOH, PAMAM and TPGS-PAMAM were recorded on NMR spectrometer (JEOL 500 MHz) using solvents such as CDCl3, DMSO-d6 and D2O. The value of chemical shift of proton is denoted in ppm (δ). The degree of grafting is calculated by following equation, whereas S represents the degree of conjugation in equation.

\[ S = \frac{\text{integral areas of } -\text{CH}_3 \text{ at } 0.8 \text{ ppm in TPGS}}{\text{integral area of } -\text{CH}_2\text{CO} \text{ in PAMAM at } 2.3 \text{ ppm}} \]  

Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF)

The mass of unconjugated PAMAM and TPGS-PAMAM dendrimer samples were determined using MALDI-TOF (AB Sciex 4800 plus spectrophotometer). Beta indole acrylic acid was used as matrix in MeOH/water solvent and 10 wt % of the dendrimer samples were prepared.
**Fourier transform infrared red spectroscopy (FTIR)**

In order to understand functionalisation between TPGS and PAMAM G4 dendrimer, infra-red spectroscopy was used. The spectra of TPGS, TPGS-COOH, PAMAM and TPGS-PAMAM samples were analysed on FTIR (Bruker, USA) instrument based on attenuated total reflection mode. The pre-dried samples were placed under diamond tip for analysis and recording of spectra.

**Quantification of free amine by TNBS assay**

To quantify number of TPGS-COOH attached with primary amines, group on PAMAM was analysed through TNBS assay. The extent of conjugation was evaluated by the difference in amount of free amine between TPGS conjugated PAMAM and unconjugated PAMAM. The standard curve is prepared by using aqueous TNBS solution of 25 μg/mL was added to the different concentrations (25–200 μg/mL) of PAMAM sample. Absorbance of the samples was analysed using UV–visible spectrophotometer (Agilent carry system). The well containing TNBS reagent was considered void sample to measure the baseline absorbance. For determination of TPGS-PAMAM concentration, carrier (C) (μg/mL) per well was acquired by interpolation of the calibration equation of PAMAM [32]. The percentage conjugation (X) for TPGS-PAMAM NPs was calculated through following equation:

\[
C(64 - X) = n[(14214 + (X + S)]
\]

(2)

The amount of primary amine groups on PAMAM G4 generation dendrimer is 64, therefore to quantify the free amine groups on each PAMAM carrier is denoted (n) and the M.W of attached substituent TPGS-COOH is 1613.12 Da (S).

**Differential scanning calorimetry (DSC)**

For thermal analysis of PAMAM, TPGS-COOH and TPGS-PAMAM were done through DSC (Thermo analytical Instrument, USA). Precisely weighed samples were sealed into aluminium T-zero pans. The ramp temperature for samples was set at 5 °C/min ranged from 0 °C to 180 °C. During analysis, the flow of ultrapure nitrogen gas 50 mL/min was maintained in sample holding chamber [33].

**UV-visible spectroscopy**

The UV absorption spectra of the PIP-TPGS-PAMAM, PIP and TPGS-PAMAM were recorded. Using 100.0 μL samples were diluted in 1.0 mL methanol solutions. The spectra were measured using a UV-visible spectrophotometer (LAB INDIA-3000) using cuvette of quartz having an optical path length of 10.0 mm. Methanol was used to subtract the background noise [34].

**Particle size and zeta potential determination**

The hydrodynamic size and zeta potential of PIP-TPGS-PAMAM and PIP-PAMAM were determined using Zetasizer (Malvern Nano-ZS UK). Nanoparticle suspension was diluted 10 times with TDW prior to analysis. The responses were measured in triplicate (n = 3) [35].

**High resolution transmission electron microscopy (HR-TEM)**

The particle size and morphology of PIP-TPGS-PAMAM and PIP-PAMAM were analysed using HRTEM (JEOL, Tokyo, Japan) on 200 kV voltage. The nanosuspension of dendrimers was diluted to 1 mg/mL and were placed on carbon coated grid and dried at room temperature prior to measurements [36].

**Estimation of drug content**

The amount of PIP encapsulated in dendrimers was analysed through high-performance liquid chromatography (HPLC) (Waters, USA) attached with photodiode array detector and reverse phase C18 column. Methanol and water were used as mobile phase at 60:40 with flow rate of 1.0 mL/min and column temperature was set at 25 ± 0.5 °C. The injection volume adjusted to 20 μL at 343 nm wavelength for 7.4 min retention time. The calibration curve of PIP from 0.5 to 20 μg/mL concentration range showed regression coefficient of 0.992. The percentage encapsulation efficiency (% EE) was analysed through dialysis method. The 2 mL of PIP-TPGS-PAMAM and PIP-PAMAM nanosuspension was placed into a dialysis bag having 12 kDa MW cut off. The nanosuspension was dialysed against TDW (1000 mL) at room temperature for 4 h to remove the free PIP [37]. To extract PIP from PIP-TPGS-PAMAM, nanocarrier was solubilised in methanol and ultrasonicated for the removal of encapsulated PIP and filtered through 0.2 μm pore size syringe filter prior to HPLC analysis. The %EE of PIP in TPGS-PAMAM dispersion was obtained from following equation:

\[
\% \text{EE} = \frac{\text{Concentration of PIP in dendrimer}}{\text{Total Weight of PIP Added in dendrimer}} \times 100
\]

Whereas percentage drug loading was calculated after lyophilisation of PIP-TPGS-PAMAM using 1% mannitol. The amount of PIP is estimated after reconstituting the briefly weighed powder in methanol and analysed on HPLC.

\[
\% \text{DL} = \frac{\text{Amount of Drug}}{\text{Weight of Dendrimer}} \times 100
\]

**Stability studies of PIP-TPGS-PAMAM**

The stability of the PIP-TPGS-PAMAM dendrimers was conducted at room temperature in phosphate buffer pH 7.4 for 30 days. The zeta potential and particle size for PIP-TPGS-PAMAM were monitored using DLS [38].

**In-vitro studies**

**In-vitro drug release**

The PIP release study from PIP-TPGS-PAMAM, PIP-PAMAM and free PIP was investigated through dialysis technique. Briefly, free PIP, PIP-TPGS-PAMAM and PIP-PAMAM containing equal amount 3 mg of pristine PIP was introduced into bag of dialysis membrane having MW cut off 12,000 Da. The release of PIP was conducted in 50 mL PBS of pH 7.4 containing tween-80 1% w/v at 37 ± 0.5 °C temperature. The media was continuously agitated at 100 rpm on magnetic stirrer. For analysis, samples were withdrawn at scheduled time period and replenished with the equivalent volume of fresh PBS medium to maintain the sink conditions. The amount of PIP in samples was determined through HPLC. The percentage cumulative amount of drug released (% CDR) was determined through the following equation [39].
% CDR = \frac{\text{Amount of PIP Released at Specific Time}}{\text{Total Amount of PIP in Dendrimers}} \times 100 \quad (5)

Antioxidant activity studies

DPPH assay was done to investigate the antioxidant property of Free PIP, TPGS-PAMAM and PIP-TPGS-PAMAM. These formulations were diluted with methanol to obtain concentration of 1 mg/mL stock solution. The stock samples were serially diluted to prepare different concentrations range of 10, 50 and 100 \mu g/mL in PBS. The diluted samples were mixed with an equal volume of DPPH methanolic solution (100 \mu M) followed by incubation for 30 min in a dark place. The absorbance of samples was recorded at 517 nm using microtiter plate reader and DPPH solution without sample was considered as control. % Antioxidant activity of samples was calculated using the formula:

\% \text{Antioxidant activity} = \frac{A_{\text{DPPH}} - A_{\text{Sample}}}{A_{\text{DPPH}}} \times 100 \quad (6)

where \( A_{\text{DPPH}} \) and \( A_{\text{Sample}} \) are the absorbances of DPPH without and with samples, respectively. All the experiments were performed in triplicate [40].

\( A_{\beta1-42} \) monomer preparation

For preparation of \( A_{\beta1-42} \) monomers, lyophilised powder was dissolved in HFIP using microcentrifuge tube. The HFIP was removed using high vacuum apparatus from \( A_{\beta1-42} \) solution. The film of \( A_{\beta1-42} \) peptide forms at inner wall of microcentrifuge tube which was stored at \(-20^\circ\text{C}\) temperature. DMSO (Dimethylsulphoxide) was used to dissolve the \( A_{\beta1-42} \) film and diluted in PBS to obtain appropriate concentration [41].

\( A_{\beta} \) fibril disaggregation ThT assay

For disaggregation studies the \( A_{\beta1-42} \) solution (10 \mu M) was incubated at 100 rpm for 72 h at 37 \^\circ\text{C} to attain the sufficient amount of \( A_{\beta1-42} \) aggregates [29]. To monitor the disaggregation of \( A_{\beta1-42} \) fibrils, thioflavin-T (ThT) (10 \mu M) was used as a probe. The equivalent concentration of PIP (50 \mu g/mL) was used in PIP-TPGS-PAMAM, free PIP and void TPGS-PAMAM added into the preformed \( A_{\beta1-42} \) aggregates solutions with ThT for further incubation. The absorption of ThT reagent was measured at 480 nm emission and 450 nm excitation. The measurement was done on ELISA plate reader (synergy) and experiments were done in triplicates.

\( A_{\beta} \) monomer kinetics ThT assay

In fibrillation, kinetics studies \( A_{\beta1-42} \) (10 \mu M) monomers were incubated with group of PAMAM, PIP-PAMAM, void PIP-PAMAM, PIP-TPGS-PAMAM, free PIP and TPGS-PAMAM containing (50 \mu g/mL PIP) for 72 h. For this study at predetermined time interval, fluorescence was analysed on ELISA plate reader. The fluorescence of ThT reagent was set at 480 nm emission, and 450 nm excitation wavelength and the experiments were done in triplicates.

\( A_{\beta1-42} \) disaggregation monitoring using DLS

DLS was used to monitor particle size distribution of co-incubated \( A_{\beta1-42} \) fibril with PIP-TPGS-PAMAM, void TPGS-PAMAM and PIP. The samples were dilute 10 folds in triple distilled water prior to analysis, and measurements were taken in triplicates [42].

Circular dichroism spectroscopy (CD)

The CD spectra of final \( A_{\beta1-42} \) aggregates (10 \mu M) incubated with free PIP, PAMAM, void TPGS-PAMAM, PIP-PAMAM and PIP-TPGS-PAMAM containing 10 \mu M equivalent of PIP monitored after 72 h. The spectra of samples were measured at scan rate of 1 nm/sec from range 190 to 250 nm recorded on (JASCO J810, Tokyo Japan) spectropolarimeter. Constant nitrogen purging was maintained during analysis of the sample [43].

Atomic force microscopy (AFM)

The PIP-TPGS-PAMAM incubated with \( A_{\beta1-42} \) aggregate for 12, 48 and 72 h samples were applied over freshly cut mica (1 cm²) of sheet and incubated for 3 min at room temperature. The nanocarrier suspension was then washed with water to remove any traces of residues and dried using nitrogen gas. A Nanoscope V Multimode scanning probe was used to capture the images of samples.

Scanning electron microscopy (SEM)

The \( A_{\beta1-42} \) disaggregation was also monitored using SEM (JEOL), PIP-TPGS-PAMAM incubated \( A_{\beta1-42} \) fibrils were dried and sputter coated with gold layer. After placing the sample in vacuum chamber at 3 kV, the images of samples were observed at 12, 48 and 72 h.

In-vitro cell line studies

Cytotoxicity assays

SHSY5Y cells were procured from national centre for cell sciences (NCCS Pune), which was grown in DMEM. The cell culture medium includes 10% foetal bovine serum, 1% streptomycin and incubated in 5% CO2. The \( A_{\beta1-42} \) fibril-induced cytotoxicity studies were performed on SHSY5Y cells using MTT reagent. The cells were seeded in 96-well plate having cell count of \( 1 \times 10^4 \) cells/well which are incubated for 24 h to adhere in wells. Fresh culture media was added in cells prior to treatment with \( A_{\beta1-42} \) precultured fibrils (10 \mu M). Then cells are treated with PBS (control), \( A_{\beta1-42} \) (10 \mu M) (negative control), void TPGS-PAMAM + \( A_{\beta1-42} \) (10 \mu M), PIP (PIP concentration 10, 50 and 100 \mu g/mL) + \( A_{\beta1-42} \) (10 \mu M), and PIP-TPGS-PAMAM (PIP concentration 10, 50 and 100 \mu g/mL) + \( A_{\beta1-42} \) (10 \mu M). Cells were incubated in CO2 incubator after treatment for 24 h at 37 \^\circ\text{C}. Cells were further washed with PBS prior to addition of MTT reagent (10 \mu L) with concentration 4 mg/mL. After 4 h, incubation cells were treated with 100 \mu L DMSO to dissolve formazan crystals. ELISA microtiter plate reader (synergy model) was used to analyse the absorbance of MTT reagent in cells at 570 nm. Results were shown in % cell viability [8]. Experiments were performed in triplicates for statistical significance analysis of variance was implemented.

\% \text{Cell Viability} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100 \quad (7)

Determination of the intracellular ROS levels

H2DCF-DA reagent was used to analyse the amount of free radical concentration in \( A_{\beta1-42} \) treated cells [44]. The SHSY5Y cells were grown in six-well plate for 24 h with coverslip prior to treatment with \( A_{\beta1-42} \) (10 \mu M) and formulation. The treatment groups were
divided in control (PBS), negative control (Aβ₄₂ only), free PIP (50 μg/mL) + Aβ₄₂ (10 μM), void TPgs-PAMAM + Aβ₄₂ (10 μM) and PIP-TPgs-PAMAM (50 μg/mL) + Aβ₄₂ (10 μM). Then cells were incubated in serum-free medium for 24 h at 37°C. Then cells were rinsed using PBS and treated with H₂DCF-DA and again washed with media. Fluorescence of H₂DCFDA was measured through flow cytometer (Cytoflex Beckman Coulter) on excitation/emission wavelength 485 nm/535 nm.

Intracellular Aβ aggregate detection

SHSY5Y cells were grown in six-well plate and treated with control (PBS), negative control (Aβ₄₂ only), free PIP (50 μg/mL) + Aβ₄₂ (10 μM), void TPgs-PAMAM + Aβ₄₂ (10 μM) and PIP-TPgs-PAMAM (50 μg/mL) + Aβ₄₂ (10 μM). Then cells were incubated in serum free medium for 24 h at 37°C. Then cells were fixed in 4.0% paraformaldehyde for 10 min at room temperature. The cells was incubated for 30 min with a 0.05% solution of ThT and DAPI (1 μg mL⁻¹) and washed to remove any excess stain. The images of cells were taken on fluorescence microscopy (Olympus, Japan) at 40× magnification [44].

Apoptosis studies

For apoptosis studies, SHSY5Y cells were grown in six-well plate and treated with control (PBS), Aβ₄₂ (10 μM), PIP (50 μg/mL) + Aβ₄₂ (10 μM) and PIP-TPgs-PAMAM (50 μg/mL) + Aβ₄₂ (10 μM) incubated for 24 h. The cells were harvested after 24 h of incubation, washed twice in PBS, collected and resuspended in 500 μL of binding buffer prior to staining. The cells were incubated with Annexin V fluorescence isothiocyanate (FITC) (5 μL) and propidium iodide (PI) (10 μL) for 20 min in the dark and analysed flow cytometry (Cytoflex Beckman Coulter) [45,46].

Statistical analysis

The data were shown in standard deviations (SD±), and data were statistically analysed using variance analysis (ANOVA) as appropriate, (−) p < .01 and (+) p < .05, respectively, indicating significant differences between the treated and control sample.

Results and discussion

This PAMAM dendrimer was grafted with TPgs to reduce the toxicity and increase biocompatibility without hampering in-vitro Aβ₄₂ disaggregation activity. The hypothesis for the fabrication of TPgs-PAMAM was used to encapsulate and deliver PIP in neuronal cells with reduced Aβ₄₂-induced toxicity.

Synthesis and conjugation of TPgs-PAMAM

The functionalisation of TPgs with PAMAM is two-step process, the first step involves conjugation of succinic ester to TPgs for introduction of carboxylate group shown in a schematic representation (Figure 1). In second step, free amines at periphery of PAMAM G4 enable the formation of amide bond with TPgs-COOH to form TPgs-PAMAM. The conjugation of TPgs with succinic anhydride was done using DMAP which is weak base that act as catalyst for the ring-opening and esterification. TPgs was reacted with excess amount of SA to avoid crosslinking with PAMAM. The proton NMR analysis of TPgs-COOH showed the succinyl methylene peaks at 2.5–2.6 ppm and δ-tocopherol signal at 0.8 ppm represented in Figure 2(a,b). The succinoylation is confirmed by integration of the proton peaks at 3.5 ppm polyethylene glycol peak and supporting table ST [30].

The internal amides of PAMAM dendrimer were observed at 5.2 ppm shown in Figure 2(c) and supporting table ST, and the methylene peaks of dendrimer adjacent to amide were observed from 2.3 ppm. PAMAM G4 free amines were attached with free carboxylic acid through EDC chemistry coupling reaction to form an amide bond between TPgs-COOH and PAMAM dendrimer. The spectra of TPgs-PAMAM H¹ NMR were recorded in DMSO-d₆ which exhibited characteristic peak of TPgs at 0.8 (–CH₃), 1.0–1.5 (–CH₂–CH₂), 3.5–3.7 (–CH₂CH₂O) and for PAMAM at 2.30–2.50 (–CH₃CONH), 2.62–2.73 (–CONHCH₂CH₂N), 2.82–2.92 (–NCH₂CH₂CONH) and 3.26–3.37 (–CONHCH₃ and –CH2NH₂) ppm. The amide coupling proton of TPgs-COOH and peripheral amines
of PAMAM was observed at 7.82–8.06 ppm in Figure 2(d). The amount of TPGS group grafted in TPGS-PAMAM was calculated from the integral ratio of the signal at 2.3 ppm exhibit the $-\text{CH}_2$ adjacent to the C=O group of PAMAM and $-\text{CH}_3$ proton in the TPGS at 0.8 ppm. The reaction of TPGS-COOH with PAMAM was done at two different molar ratios such as 25:1 which showed 12 TPGS molecules attached to dendrimer. At reactant molar ratio of 50:1, integral ratio calculation exhibited 20 TPGS molecule is conjugated to per PAMAM dendrimer. The TPGS-COOH reaction efficiency with PAMAM G4 dendrimer was found to be 40%.

FTIR and DSC
Spectra of TPGS-COOH in comparison to TPGS showed a peak at C=O at 1750 cm$^{-1}$ and sharp peak of carboxylic acid $-\text{OH}$ was observed at 3200 cm$^{-1}$ shown in Figure 3(a) [32]. However, peak observed at 1700 cm$^{-1}$ in TPGS is an ester carbonyl stretch, which also observed in TPGS-COOH. Whereas the spectra of TPGS-PAMAM showed disappearance of C=O carboxylic at 1750 cm$^{-1}$ and appearance of C=O amide stretch at 1618 cm$^{-1}$ showed successful conjugation of TPGS-PAMAM. The intensity of peak at 3400 cm$^{-1}$ is an indicator for enhancement of secondary $-\text{NH}$–amides in the PAMAM.

The formation of covalent bond between polymers moieties affects the melting point of compound, which can analysed using DSC [33]. The DSC thermogram of PAMAM displayed the sharp endothermic melting point peak (Tm) at 144.55 °C and glass transition temperature peak (Tg) at 57.23 °C. However, the TPGS-COOH showed Tm at 25.34 °C and Tg at 102.45 °C exhibited Figure 3(b). While physical mixture of TPGS-COOH and PAMAM had not showed any major shift in the melting point. Conversely, TPGS-PAMAM a new Tm peak at 110.25 °C was found with shift in Tg to 89.35 °C indicated the successful conjugation of PAMAM with TPGS-COOH.

MALDI-TOF
MALDI-TOF reflectron mode exhibits higher resolution which is used to analyse the purified product samples of G4 generations dendrimers and high MW polymers up to the mass of 100,000 Da [44]. The MW of marketed TPGS is 1513.55 Da approximately, after reaction with succinic anhydride the weight increased to 1613.25 Da for TPGS-COOH which is predicted by CHEM BIODRAW Software. PAMAM dendrimer exhibited 14214.18 m/z molecular weight through MALDI-TOF analysis. Due to MW >10,000 Da the mass of TPGS-PAMAM and PAMAM were determined using on linear delayed extraction mode displayed in Supporting Figure S1. Due to high molecular weight, the mass spectra of PAMAM and TPGS-PAMAM m/z peaks cover a broad region ± 10,000 units in mass spectra. At reaction molar ratio of 25:1 (TPGS-COOH: PAMAM), the mass of TPGS-PAMAM was found to be 35183.25 m/z indicated 12 TPGS-COOH groups are attached per PAMAM molecule. Further reaction at different molar ratios between TPGS-COOH and PAMAM was estimated through TNBS assay.

TNBS assay and dendrimer conjugate zeta potential
In this assay, primary amine displaces the sulphonic acid group on TNBS reagent and shows absorbance at 410 nm [47]. Reaction at molar ratio 25:1 and 50:1 of TPGS-COOH to PAMAM in second step showed attachment of 12.19 to 20.12 TPGS molecules per PAMAM dendrimer shown in Table 1. The attachment of TPGS-COOH reduces the absorbance of TNBS reagent due to reduction in the amount of TNBS reagent used.
in number of free amine sites at PAMAM dendrimer surface. This reduction in number of free amines at PAMAM due to grafting, which resulted in shift of zeta potential from 11.33 mV to −6.05 mV. The TPGS groups at periphery of TPGS-PAMAM shield the positive charge of free amine and leads to reduction in zeta potential from positive to negative. The molar ratio 50:1 TPGS-PAMAM was selected for further studies due to higher TPGS surface attachment, and this shielding of PAMAM free amine can reduce the fabricated nanocarrier cytotoxicity.

HR-TEM analysis
The morphology of PIP-TPGS-PAMAM and PIP-PAMAM was characterised using HR-TEM. Granular spherical-shaped particle of PIP-PAMAM was observed having particle size of 15.42 nm in Figure 4(a). But the morphology of PIP-TPGS-PAMAM observed to be spherical and rigid with an average particle diameter less than 50 nm displayed in Figure 4(b). meanwhile, the size of grafted dendrimer was also investigated by DLS which indicated that hydrodynamic diameter detected averagely 43.0 nm showed in Figure 4(c) which was approximately similar comparison to TEM micrographs. Due to TPGS, attachment on peripheral amines of PAMAM increased the MW and size of PIP-TPGS-PAMAM in comparison to PIP-PAMAM. However, HRTEM imaging revealed PIP-TPGS-PAMAM is monodisperse in nature.

Physical characterisation of conjugated dendrimers
The encapsulation of PIP in TPGS-PAMAM was done through solvent injection method to form PIP-TPGS-PAMAM. Methanol evaporation from the organic phase leads to the formation of PIP encapsulated PIP-TPGS-PAMAM due to nanoprecipitation. Aqueous solubility of free PIP is reported to be poor which is not favourable for achieving drug concentration efficacy at target site [6]. The internal structure of the dendrimer is usually non-polar due to the combination of hydrophobic and hydrogen bond formation enables incorporation of lipophilic drugs in PAMAM matrix. PIP-PAMAM showed only 73.23% EE; however, TPGS conjugation with PAMAM increases the % EE to 80.35% for PIP which is 10% higher than PIP-PAMAM shown in Table 1. This increment in entrapment is due to tocopherol-mediated hydrophobicity in TPGS-PAMAM compared to non-conjugated PAMAM [48,49]. DLS and zeta potential results revealed the particle size and surface charge characteristics of dendrimers. DLS showed 15.32 nm hydrodynamic radius of PIP-PAMAM, and zeta potential was found to be 11.5 mV data provided in Table 1. After TPGS grafting with PAMAM dendrimer, particle size of PIP-TPGS-PAMAM increased to 43.23 nm with reduction in zeta potential to −6.2 mV shown in Supporting Figure S2a. The macromolecular nature of TPGS provides steric stability of nanocarriers and polyethylene glycol group of TPGS forms hydrogen bonding with water molecules in aqueous medium [27].

For PIP-PAMAM, the % DL was found to 6.8%, whereas the TPGS grafting with PAMAM increased the PIP loading to 10.3% shown in Table 1. Increment of the hydrophobic moiety such as tocopherol group in the nanocarrier enhanced the encapsulation of PIP. The entrapment of PIP in TPGS-PAMAM matrix was analysed by UV-visible spectrophotometer. The comparative absorption spectra of TPGS-PAMAM, PIP-TPGS-PAMAM and PIP were measured for their lambda max peaks shown in Figure 4(d). It was observed that TPGS-PAMAM and PIP showed intense absorption peak at 281 nm and 345 nm. In the spectra of PIP-TPGS-PAMAM dendrimer, the intensity is reduced peaks at 345 nm compared to free PIP which showed the encapsulation PIP having equivalent concentration of (5 µg/mL). The PIP containing PIP-TPGS-PAMAM was observed at different concentration (1, 5, and 10 µg/ml) in methanol shown in Supporting Figure S2b.

Stability studies and in-vitro drug release
The 30 days storage stability study of PIP-TPGS-PAMAM dendrimer was monitored via measuring particle size and zeta potential using DLS at room temperature. The particle size of nanocarriers were increased from 40 ± 2 nm to 48 ± 3 nm in one month of storage at room temperature indicated no drastic stability issue. However, zeta potential increased from −6.2 mV to −4.5 mV without change in visible stability of PIP-TPGS-PAMAM presented in Figure 5(a,b). TPGS-PAMAM showed −6.2 mV zeta potential, but TPGS is a macromolecular neutral amphiphilic structure which that sterically stabilises PAMAM dendrimers and prolongs the stability of nanocarriers [27].

The % CDR of PIP from the PIP-PAMAM and PIP-TPGS-PAMAM dendrimer was observed at cerebrospinal and physiological pH 7.4. The release pattern of PIP was biphasic which includes an early phase with burst release and a late phase of sustained release in case of PIP-TPGS-PAMAM. The 30.98% of PIP was released from the TPGS-PAMAM matrix in the first 8 h. When the second phase-initiated PIP-TPGS-PAMAM dendrimer released 69.85% PIP in 96 h. Due to lipophilic
nature free PIP showed release of 27.28% of PIP in dissolution media compared to PIP-TPGS-PAMAM. Whereas PIP-PAMAM exhibited 80.19% PIP release from dendrimer matrix to media in 96 h shown in Figure 5(c). This indicates the aqueous solubility of PIP increased after encapsulation in dendrimers compared to free drug. The rigidity of PIP-TPGS-PAMAM increased due to hydrophobic tocopherol component in aqueous environment that restricted the release of PIP from core branches of dendrimer. TPGS-COOH is conjugated with PAMAM through amide bond which is not prone to hydrolysis and maintains sustained release of drug [10,50]. The HPLC chromatogram showed only retention peak of PIP at 7.3 min, no other degradation peak was observed during release studies shown in Supporting Figure S3. This indicates PAMAM matrix, protects PIP from degradation and provides stability to nanocarrier.

Table 1. (a) TNBS assay-based degree of conjugation and zeta potential of dendrimers; (b) physical characteristics of PAMAM dendrimer conjugates; (c) cell viability studies of dendrimer conjugates against amyloid toxicity.

(a): TNBS assay-based degree of conjugation and zeta potential of dendrimers

| Formulation   | Molar ratio of TPGS-COOH/PAMAM in reaction | Zeta potential (mV)±SD | No. of TPGS attached per PAMAM dendrimer |
|---------------|--------------------------------------------|------------------------|------------------------------------------|
| PAMAM-G4      | –                                          | 11.33±0.4              | –                                        |
| TPGS-PAMAM    | 25:1                                       | -1.5±0.8               | 12.19                                    |
| TPGS-PAMAM    | 50:1                                       | -6.2±0.6               | 20.12                                    |

(b): Physical characteristics of PAMAM dendrimer conjugates

| Batches       | Piperine to dendrimer ratio | Particle size (nm) ± SD | Zeta potential (mV) ± SD | PDI | % EE (%)± SD | DL (%) ± SD |
|---------------|----------------------------|-------------------------|--------------------------|-----|-------------|-------------|
| PIP-PAMAM     | 1:10                       | 15 ± 4                  | 11.5±0.2                 | 0.201| 73 ± 3.3%   | 6.8 ± 2.3%  |
| PIP-TPGS-PAMAM| 1:10                       | 43 ± 2                  | -6.2±0.4                 | 0.195| 80 ± 3.5%   | 10.3 ± 2.9% |

(c): Cell viability studies of dendrimer conjugates against amyloid toxicity.

| Treatments on SHSY5Y cells | PIP conc. (10 μg/mL) | PIP conc. (50 μg/mL) | PIP conc. (100 μg/mL) |
|-----------------------------|----------------------|-----------------------|------------------------|
| Free PIP + Aβ 1-42 (10 μM)  | 57 ± 2.3             | 62 ± 3.5              | 67 ± 1.5               |
| PIP-TPGS-PAMAM + Aβ 1-42 (10 μM) | 69 ± 3.3           | 73 ± 2.6              | 82 ± 3                 |
| TPGS-PAMAM + Aβ 1-42 (10 μM) | 49 ± 3.7             | 37 ± 2.8              |                        |
| Aβ 1-42 (10 μM)             |                      |                       |                        |
| Control                     | 100                  |                       |                        |

Figure 4. TEM micrograph of (a) PIP-PAMAM and (b) PIP-TPGS-PAMAM. Particle size of PIP-TPGS-PAMAM, and PIP-PAMAM (c). UV-visible spectrum of PIP, PIP-TPGS-PAMAM and TPGS-PMAM (d).

DPPH assay

DPPH assay offers the quantitative estimation of antioxidant potential for radical scavenging compounds [40]. ROS have been described to induce cell death through apoptosis in neuronal cells which can be inhibited via broad range of intra and extracellular antioxidants [51]. PIP and TPGS are known for their antioxidant activity, which are incorporated in the fabricated nanocarrier and are further analysed for free radical scavenging activity. The antioxidant activity of free PIP was observed 28.25±3.4%, whereas due to tocopheryl group the activity increased for TPGS-PAMAM to 54.15±4.3% at 100 μg/mL concentration shown in Figure 5(d). The PIP-TPGS-PAMAM showed the enhanced antioxidant activity ≥80% in comparison to free PIP. PIP-TPGS-PAMAM enhanced the direct antioxidant activity against free radicals and the potential...
efficacy of PIP to protect cells from oxidative damage. Overall, PIP-TPGS-PAMAM was found to enhance the free radical scavenger activity compared to free PIP.

**ThT assay for Aβ fibril disaggregation**

Aβ_{1-42} aggregation is a protein conformational disorder which transforms the Aβ_{1-42} peptide from monomeric α-helical to β-pleated sheets structure [28,52]. This Aβ aggregation was monitored using ThT reagent which binds with Aβ fibrils to exhibit hypsochromic shift [41]. For ThT assay of Aβ_{1-42}, aggregates were prepared and diluted in PBS. Then Aβ_{1-42} (10 μM) fibrils were co-incubated with ThT dye and divided into treatment groups such as free PIP, void TPGS-PAMAM and PIP-TPGS-PAMAM. The fluorescence intensity of only Aβ_{1-42} as free PIP, void TPGS-PAMAM and PIP-TPGS-PAMAM. The fluorescence intensity of only Aβ_{1-42} group showed 3323 ± 277 au after 72 h incubation. Those Aβ_{1-42} groups which were treated with PIP-TPGS-PAMAM dendrimer showed remarkable reduction in fluorescence to 764 ± 115 au compared to other group in 72 h as shown in Supporting Figure S4(a). The TPGS-PAMAM also reduced the fluorescence, but the PIP-TPGS-PAMAM showed distinct Aβ_{1-42} disaggregation. The groups treated with free PIP showed weak reduction of the ThT fluorescence, whereas TPGS-PAMAM reduced the ThT fluorescence intensity to 1435 ± 427 au. The disaggregation of pre-cultured fibrils was observed highest in groups, which are incubated with PIP-TPGS-PAMAM. Encapsulation of PIP in TPGS-PAMAM increased the anti-amyloidgenic activity than other treatment groups. PIP and PAMAM both are Aβ fibril inhibitor and combining them in a single nanocarriers PIP-TPGS-PAMAM synergised the activity. PEG is known to have more H-bond sites, which may allow it to engage with Aβ fibrils more effectively [42].

**ThT assay for Aβ monomer fibrillation**

The prepared monomers of Aβ_{1-42} were assessed for the fibrillation kinetic assay as shown in Figure 6(a). The highest fibrillation rate was found with the group of nontreated monomers exhibited 6749 ± 483 au fluorescence in 72 h. The groups which are treated with PIP-TPGS-PAMAM showed highest reduction of fluorescence to 2642 ± 525 au in 72 h. The group treated with TPGS-PAMAM showed the distinct inhibition of fibril growth. TPGS-PAMAM ThT fluorescence reduced to 4031 ± 399 au in 72 h. Whereas PIP treated group exhibited fluorescence intensity to 5895 ± 366 au indicating non-significant Aβ_{1-42} fibril growth inhibition. PIP is water insoluble molecule and unable to interact with Aβ_{1-42} fibrils in aqueous medium, but PIP encapsulated PIP-TPGS-PAMAM increases the payload to Aβ_{1-42} fibrils. Therefore, PIP-TPGS-PAMAM showed the highest fibril inhibition compared to other groups. The PEG negative charge associated with PIP-TPGS-PAMAM inhibits the further aggregation of the Aβ due to electrostatic interaction.

**Aβ disaggregation monitoring using DLS**

To validate Aβ_{1-42} fibril disaggregation of ThT assay DLS was used to determine the particle size of aggregates. Prior DLS analysis these fibril were incubated dendrimer conjugate for 72 h. DLS is normally used to evaluate the hydrodynamic diameter of particles in solution and the % intensity of scattered light is affected by larger size particle in aqueous dispersion. The Aβ_{1-42} fibril has heterogeneous rod like structure from 100 to 10,000 nm, therefore DLS is used to obtain the qualitative estimation of aggregate particle size [52]. Particle distribution of Aβ_{1-42} exhibited four main peaks of outsized particles with the diameters of ~100 nm, ~800 nm, ~>5 μm and 10 μm with PDI 0.65 shown in Supporting Figure S5b. Prior measurement of particle size of PIP-TPGS-PAMAM showed 43 ± 2.5 nm diameter which was incubated with Aβ_{1-42} aggregates of 10 μM for 72 h. Free PIP showed the small extent reduction in particle size of Aβ aggregates intensity and PDI reduced from 0.655 to 0.532. Those Aβ aggregates group which are incubated with void TPGS-PAMAM showed the reduction of particle size to 700 nm and decreased PDI from 0.6 to 0.3 for aggregates. However, group treated with PIP-TPGS-PAMAM showed of Aβ_{1-42} aggregates were reduced to smaller fragments and a monodisperse peak observed 63 nm. These smaller fragments of Aβ_{1-42} have particle size distribution ~PDI of 0.2122 showed highest disaggregation of Aβ due to PIP-TPGS-PAMAM. Reportedly, surface modified PAMAM dendrimers are inhibitors of Aβ_{1-42} aggregates via electrostatic interaction blocks the growth of the terminal points and increases the fibril breakage rate [25].
Therefore, the PIP-TPGS-PAMAM NPs showed higher Aβ fibrillation inhibitory effect compared to the other groups. However, encapsulation of PIP in TPGS-PAMAM increased the payload to Aβ1-42 fibrils, therefore PIP-TPGS-PAMAM showed highest fibril disaggregation property. DLS analysis revealed large Aβ1-42 aggregates reduced to smaller amorphous species (<100 nm) by PIP-TPGS-PAMAM which is crucial to mitigate neurotoxicity in cells.

**CD conformational analysis**

Furthermore, the effect of fabricated dendrimer on Aβ1-42 fibril disaggregation was investigated using CD to analyse conversion of secondary β-pleated to α-helical conformation [43]. The CD spectrum measurement of PIP-TPGS-PAMAM scanned from 190 to 250 nm and showed straight line used as control which is shown in Figure 6(b). When Aβ1-42 (10 μM) aggregates showed the intense deep negative peak of β-pleated sheets of amyloid fibrils at 214 nm. Further shift of wavelength to 220 nm was observed for PIP-PAMAM and PAMAM indicator of fibril conformational reversal activity. Free PIP showed slight reduction, whereas TPGS-PAMAM reduced the β sheet intensity from −8.6 to −6.1 Δ χ (deg). Whereas fibrils incubated with PIP-TPGS-PAMAM (10 μM) exhibited negative peak at 220 nm and reduced the fibril from β sheet to α-helical structure. This reduction in peaks intensity of Aβ1-42 aggregates due to PIP-TPGS-PAMAM showed that fabricated nanocarriers are able to change the conformation from secondary β-pleated to α-helical structure; PIP-TPGS-PAMAM enhanced the conformational changes to compare to PIP-TPGS-PAMAM. The macromolecular nature of TPGS-PAMAM and PEG electrostatic charge reversed the fibril conformation [28]. ThT assay, CD and DLS characterisation revealed PIP-TPGS-PAMAM showed highest Aβ1-42 fibril disaggregation activity. Furthermore, PIP-TPGS-PAMAM incubated Aβ1-42 fibrils were studied through microscopy.

**Aβ fibril disaggregation monitoring AFM and SEM**

The nanocarrier-induced disaggregation of Aβ aggregates was monitored using AFM at predetermined intervals [53]. The PIP-TPGS-PAMAM incubated aggregates morphology at 12 h showed no significant change in fibril structure. Increment in incubation period leads to fragmentation of Aβ1-42 fibrous aggregate was observed in 48 h. After 48 h, incubation period aggregates fragmented to smaller particle size. PIP-TPGS-PAMAM disaggregated the fibrils converted to amorphous monomeric state in 72 h. The SEM was also used to monitor the disaggregation efficiency of PIP-TPGS-PAMAM on Aβ aggregates. The SEM images exhibited distinct Aβ1-42 fibrils at 12 h with PIP-TPGS-PAMAM dendrimers shown in Figure 7(a). The fibrils disaggregation was clearly observed after 48 h showed fibres were fragmented to small length amorphous aggregates. SEM and AFM images confirmed the disaggregation Aβ1-42 fibrils due to PIP-TPGS-PAMAM. We discovered that PIP-TPGS-PAMAM reduced most of the long fibrils to shorter fragments, which reformed into amorphous structures, using both AFM and SEM images.

**Cell line studies**

**MTT assay**

MTT was used to analyse the neuroprotective activity of PIP-TPGS-PAMAM against Aβ1-42 fibril-induced cytotoxicity in SHSY5Y cell [54]. Due to the neuroprotective property of PIP, the treatment was done at equivalent concentration (10, 50 and 100 μg/mL) for free PIP and PIP-TPGS-PAMAM shown in Table 1. The group of cells which are treated with only Aβ1-42 (10 μM) aggregates reduced the cell viability to 37.23% in comparison to control shown in Figure 7(b). Void TPGS-PAMAM showed viability of cells to 49.37% compared to control. The tocopherol moiety in nanocarrier inhibits Aβ1-42-induced free radicals and increases cell viability [17]. Although cells treated with PIP-TPGS-PAMAM at 10 μg/mL dose of equivalent PIP concentration, increment in cell viability to 69.23% was observed. At 100 μg/mL PIP concentration, SHSY5Y cells viability was observed to be 67.83% and 82.55% for free PIP and PIP-TPGS-PAMAM compared to control, respectively, shown in Table 1. PAMAM G4 are Aβ1-42 fibril growth inhibitors, but due to positive surface charge it induces cytotoxicity. Therefore, PAMAM surface is grafted with TPGS to reduce the cytotoxicity and deliver PIP inside the cell. Cell groups which are treated with PIP-TPGS-PAMAM at (100 μg/mL) concentration increased the cell viability to 82.55 ± 2.8% compared to only Aβ1-42 treated group. At 10 μg/mL containing PIP in PIP-TPGS-PAMAM showed significant increment in viability, therefore in further cell line studies only 50 μg/mL PIP dose used. PIP-TPGS-PAMAM, in general, transforms fibrils into spherical aggregates that have been found to be less toxic [55]. PIP-TPGS-PAMAM showed the significant reduction in the Aβ1-42-induced toxicity in the SHSY5Y cells and showed significant increment in cell viability compared to other groups.

**Estimation of intracellular ROS**

H2DCF-DA can pass through the lipophilic cellular membrane via passive gradient and cleaved by intracellular ROS to form 2′,7′-dichlorofluorescein and emits fluorescence at 535 nm [56,57]. The Aβ1-42 aggregates induce ROS from mitochondria of cells that leads to neuronal cell death [54]. The H2DCF-DA used as probe to analyse the effect of free radical scavenging using flow cytometer.
for Aβ1–42 incubated PIP, TPGS-PAMAM and PIP-TPGS-PAMAM. The neuronal cells were treated with PIP, void TPGS-PAMAM and PIP-TPGS-PAMAM (50 μg/mL equivalent PIP) shown in Figure 8. The free PIP showed the reduction in fluorescence to 51.25% compared Aβ1–42 treated groups, whereas PIP-TPGS-PAMAM showed the highest reduction of H2DCF-DA fluorescence in comparison to groups treated with only Aβ1–42 aggregates. The % fluorescence intensity of PIP-TPGS-PAMAM was observed 15.84% compared to only Aβ1–42 aggregates. The PAMAM component disaggregates pre-incubated Aβ1–42 fibrils in SHSY5Y cells, whereas PIP with TPGS part in PIP-TPGS-PAMAM furnishes the free radical scavenging activity. The tocopherol in TPGS-PAMAM is antioxidant in nature and synergises the scavenging SHSY5Y cells generated ROS species by Aβ1–42 aggregates [56].

**Detection of Aβ1–42 fibrils inside SHSY5Y cells by a fluorescence microscope**

To monitor Aβ fibril in the SHSY5Y cells, ThT fluorescence observed in presence of free PIP, TPGS-PAMAM and PIP-TPGS-PAMAM. As earlier described, ThT dye binds with Aβ fibrils to emit fluorescence which estimates the amount of aggregation inside the cells. In Figure 9, fibrils were much higher in Aβ1–42 alone treated cells, whereas the group cells were co-incubated with PIP or void TPGS-PAMAM reduced the fluorescence intensity of ThT reagent but not completely. However, PIP-TPGS-PAMAM incubated group showed remarkable reduction in the fluorescence of ThT. ROS inhibition study have pointed out that both free PIP and void TPGS-PAMAM have antioxidant activity but less effective against Aβ1–42 fibril reduction. In case of PIP-TPGS-PAMAM, Aβ1–42 fibril disaggregation was enhanced due to entrapment of PIP in dendrimer matrix; however, PIP has inhibitory effect on self-aggregation of Aβ which was earlier observed in ThT assay. Furthermore, TPGS enhanced the cellular uptake of nanocarriers to disaggregate the fibril inside the neurons.

**Apoptosis studies**

The ROS species induces apoptosis in neuronal cells to further evaluate the anti-apoptotic effect of PIP-TPGS-PAMAM. Further for quantitative estimation, cells were analysed by Annexin-FITC apoptosis reagent through flow cytometer, FITC bind with membrane phospholipid of apoptotic cell and propidium iodide attaches to nucleic acid. Dot plots of flow cytometer showed PIP-TPGS-PAMAM treated cells reduced to early apoptosis 4.8%, whereas Aβ1–42 treated group exhibited 54.8% early and 1.3% late apoptosis (Figure 10). In comparison to free PIP and void TPGS-PAMAM, the PIP-TPGS-PAMAM reduced the Aβ1–42-induced apoptosis sharply. However, void TPGS-PAMAM not showed any sharp decline in apoptotic rate and exhibited 32.83% early apoptosis. This reduction programmed cell death of neurons is via dual mechanism (1) Aβ fibril disaggregation and (2) free ROS reduced by formulation. Apoptosis studies displayed the PIP-TPGS-PAMAM was able to inhibit the Aβ1–42-induced apoptosis and useful to deliver neuroprotective agent.

**Conclusion**

In summary, The PIP-TPGS-PAMAM dendrimer was fabricated through multistep approach for application for neuroprotection against Aβ fibrils. TPGS was covalently attached with PAMAM followed by physical encapsulation of PIP that led to the formation of PIP-TPGS-PAMAM dendrimers. The fabrication of the nanocarrier was confirmed by spectroscopic techniques and chemical assay. The particles size of PIP-PAMAM 15.25 nm and 43.25 nm PIP-TPGS-PAMAM respectively. Time-dependent stability showed no significant increase in particle size and PDI for PIP-TPGS-PAMAM. The in-vitro release of PIP from PIP-TPGS-PAMAM showed sustained release behaviour in 72 h at pH 7.4. PIP-TPGS-PAMAM showed significant disaggregation of Aβ fibrils by restraining the protein in its monomeric and non-toxic amorphous state. In PIP-TPGS-PAMAM nanocarrier TPGS and PIP combined antioxidant effect increased the neuroprotective activity via inhibition of radical scavenging and apoptosis activity. Our results showed that PIP-TPGS-PAMAM is possible candidate for the neuroprotection and disaggregation of amyloid fibrils. Aβ incubated with PIP-TPGS-PAMAM during Aβ fibrillisation is less toxic and can be served as nano chaperones that can prevent and re-direct Aβ fibrillisation and potential use in AD.

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Figure 8. PIP-TPGS-PAMAM preventing Aβ aggregation in SHSY5Y cells. The presence of intracellular Aβ fibrils was evaluated by ThT staining in SHSY5Y cells in the absence and presence of PIP, TPGS-PAMAM, PIP-TPGS-PAMAM and control. The cells were pre-treated with an Aβ monomer for 6 h to allow access of Aβ to the cytoplasm, sequentially incubated with PIP, TPGS-PAMAM, PIP-TPGS-PAMAM and control for an additional 24 h and visualised under a fluorescence microscope.

Figure 9. Detection of ROS production induced by Aβ1–42 fibrils in SHSY5Y cells. (A) Aβ fibrils incubated cells were incubated with absence or presence of control (a), Aβ1–42 only (b), PIP, TPGS-PAMAM (c) and PIP-TPGS-PAMAM (d) respectively. Then the samples were added to SHSY5Y cells incubated in 24 h. The ROS level was quantified by the fluorescence of DCF (488 nm excitation and 530 nm emission). (f) Quantitative analysis of the ROS level presented by the DCF fluorescence intensity. The data shown here are representative of three independent experiments with similar results.
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**Author contributions**

All authors have given permission for the final version of the manuscript.

**Disclosure statement**

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