Remarkably Efficient Blood–Brain Barrier Crossing Polyfluorene–Chitosan Nanoparticle Selectively Tweaks Amyloid Oligomer in Cerebrospinal Fluid and Aβ1–40

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Abstract: Amyloid oligomers have emerged as a key neurotoxin in Alzheimer’s dementia. Amyloid aggregation inhibitors and modulators have therefore offered potential applications in therapeutics and diagnosis. However, crossing the blood–brain barrier (BBB) and finding the toxic aggregates among aggregates of different sizes and shapes remain a challenge. The ability of identifying early aggregates can provide a new approach to find inhibitors of the initial nucleation events correlating presenile dementia. In this study, we have prepared polyfluorene nanoparticles using chitosan as an additive, which enables it to cross BBB efficiently and employed as a highly efficient amyloid oligomer modulator. The polymer conjugate, polyfluorene–chitosan (PC), shows no toxicity in MTT assay and precludes self-aggregation of Aβ1–40 and human cerebrospinal fluid oligomers to fibril formation. This modulation strategy is supported by thioflavin T assay, circular dichroism studies, atomic force microscope images, and Fourier transform infrared analysis. The polymer–protein interface exhibits the presence of co-aggregates and responded with a stable optical response. The simple synthesis to get desired sizes and shapes with necessary photophysical behavior, biocompatibility, and most prominently BBB permeability makes this polymer conjugate very unique and highly attractive for modulation of amyloid oligomers selectively as well as for developing next generation nanotheranostic materials toward presenile dementia.

Introduction

Inhibition of amyloid aggregation has emerged as one of the most crucial strategy against protein misfolding diseases. A recently developed therapy for Alzheimer’s disease, aducanumab (BIIB037), significantly lowered the amyloid burden in patients and worked like a homing device.† However, the search for structures to trap intermediates of nucleation-dependent amyloid polymerization still remains complex and challenging. Several lines of evidence indicate that Aβ may have a role in controlling synaptic activity and in the normal function of the nervous system.‡–§ Therefore, Aβ should not be considered as a mere toxic factor that needs to be eliminated to avoid the progression of the disease. It evokes neurotoxic effects during aggregation with a change in structure, shape, and size to produce senile plaques.⁶–¹² Soluble oligomers initiate disease-specific cytopathology and following symptoms whereas plaques are relatively inert but serve as pools of diffusible oligomers through dissociation.¹²–¹⁸ Therefore, identifying the small intermediates and targeting these soluble oligomers selectively may shed light on presenile dementia and therapeutic intervention. Designing efficient drugs for targeting these early aggregates/oligomers across the blood–brain barrier (BBB) makes the scenario even more complex because of the lesser surface area interaction of the higher order heterogenic aggregates and crossing the barrier efficiently.¹⁹ The BBB is embraced by endothelial cells ‘glued’ together to form junctions, and major percentage of the brain homeostasis is controlled by the entry and exit at the BBB via them. This barrier has the ability to prevent and effectively expel undesirable materials from the brain and at the same time accomplish the job of providing essential supplements, signaling molecules and the vital immune cells in the brain.²⁰–²² To tackle this dual challenge, we have designed a polyfluorene derivative to modulate early Aβ aggregates. This report details a conjugated polymer–biopolymer platform “polyfluorene–chitosan” (PC), a polyfluorene derivative (PFDDPA) and chitosan (a polysaccharide) nanocomposite which can easily cross endothelial monolayers (shown as a...
BBB mimic) unlike its precursor polyfluorene as well as modulate amyloid aggregates very efficiently. Polymerization of amyloid oligomers to final fibrils in real cerebrospinal fluid (CSF) samples as well as in commercial Aβ1-40 was examined both in the presence and absence of modulators after different time intervals by monitoring thioflavin T (ThT) fluorescent response. Secondary structure information of Aβ proteins both in the presence and absence of modulators was gathered using circular dichroism (CD) and Fourier transform infrared (FT-IR) studies. Morphological updates in the presence of modulators were collected using atomic force microscopy (AFM) images. As an additional feature, this polymeric conjugate also showed a distinct optical response in the presence of Aβ1-40 oligomers because of the preferred surface motif and further hydrophobic interaction with the hydrophobic core of the peptide that resulted in inhibition to final fibril formation. These unique observations are elaborately explained, which confirm the unique abilities of the polyfluorene nanoparticles–chitosan composites.

**RESULTS**

Alzheimer’s disease is pathologically linked to Aβ aggregation which has no physiological roles. Efforts to find efficient structural variants in either modulating these robust amyloid structures or inhibiting the aggregation process of these endogenous peptides led to the development of polymeric nanoparticles, which may lead to future theranostic precipitators. PFDP A was found to be a near perfect modulator for amyloid β (Aβ1–40) because of its hydrophobic nature; however, it failed to cross the BBB (endothelial monolayer). To overcome this, water-dispersible nanoparticles using chitosan and PFDP A were prepared (discussed in the Experimental Procedures section, Figure 1). In vitro toxicity and BBB permeability of this polymeric conjugate (PC) was confirmed prior to its use for modulation of Aβ and discussed in detail.

**In Vitro Toxicity and BBB Permeability.** Prior to the in vitro BBB assay, both the polymeric conjugate (PC) and their precursors (PFBr and PFDP A) toxicity were studied by MTT cell survival assay with red blood cells (RBCs) and Ea hy926.1, respectively (Figure 2).

Two different conjugates were prepared, namely PC2 and PC3, using both low as well as higher molecular weight chitosan along with PFDP A to assess toxicity and permeability of the conjugates.

In hemolysis assay, PFDP A and PFBr showed mild toxicity (~4–5%) to the RBC at ~100 μg/mL concentration (Figure 2a). The lower concentration was not toxic to the RBCs, whereas both the PC2 and PC3 showed lesser than 2% toxicity at the maximum concentration (100 μg/mL) (Figure 2a). In MTT cell survival assay, polymer compounds of PC2 were less than 10% toxic, whereas others were 10–30% toxic at the maximum concentration (Figure 2b). However, at 50 μg/mL and lower concentration, they did not show significant toxicity to the endothelial cells. On the basis of the toxicity assay results, 50 μg/mL concentration was used to test the in vitro BBB permeability assay (Figure 3).

The permeability efficiency of the test compounds were assessed as mentioned in the Experimental Procedures section. The endothelial monolayer permeability assay results were 46.9 ± 0.1, 18.1 ± 3.8, and 0.2 ± 0.7% movement across the barrier corresponding to PC2, PC3, and PFBr at the first hour (Figure 3b,d,f). The PFDP A concentration could not be calculated by the fluorescence method as it was sparingly soluble in culture media and gave erroneous results. Permeability efficiency data of PC2, PC3, and PFBr are tabulated in the below table. Cellular integrity in the presence of polymer conjugates were checked and remained intact throughout the experiment (Figure 3a,c,e). From the BBB secretion studies, we confirmed that polymeric conjugate (PC) was able to pass through the monolayer in a much easier manner compared to polyfluorene precursors, that is, PFBr and PFDP A. Conjugates made up of low molecular weight chitosan, PC2, were able to cross BBB more efficiently than PC3 and further utilized as a modulator (denoted as PC) in the present study (Table 1).

**Modulatory Studies Using ThT Assay.** ThT assay was prepared to examine the modulatory properties of the polymeric conjugate (PC) both in the presence of commercial Aβ1–40 as well as human CSF sample. The effects of other precursors used in making the polymeric conjugate on amyloid fibrillation (Figure 4) were also checked. First, 100 μM Aβ1–40 were incubated with 10 μg/mL PC in 10 mM phosphate buffer saline (PBS) at 37 °C and used as a stock solution. To check the effects of amyloid fibrillation, the samples were collected from the above stock solution at different time intervals starting from 0 to 84 h and diluted in 5 mM PBS. ThT (40 μM) was added to each well of the microplate reader before recording ThT emission at 482 nm using a microplate reader. Aβ1–40 (100 μM) was treated with trifluoroacetyl (TFA) and hexafluoroisopropanol (HFIP) to disaggregate as discussed in the Experimental Procedures section before incubating at 37 °C and used as a control. ThT assay of Aβ1–40 control showed a lag phase up to 50 h (Figure 4a,b, squares) before an increment in ThT fluorescence intensity because of the formation of the fibrillar network. This increase in intensity was not observed in the presence of PC (1 μg/mL, Figure 4a, circles) and the same was detected in CSF sample also (15 μM, Figure 4a, diamonds). The decrease in ThT fluorescence intensity suggested that the polymeric conjugate, PC, inhibited the formation of amyloid fibril and it was also able to disturb amyloid aggregation even in real CSF sample. Further, we have incubated PC with preformed Aβ1–40 fibrils to check the effects on already formed fibrillar networks. An
initial high fluorescence intensity at \( t_0 \) was decreased sharply and reached a plateau after 20 h of incubation (Figure 4a, triangles). To figure out the effects of other precursors which were used to prepare PC, 100 \( \mu \)M A\( \beta \)1–40 were incubated with 10 \( \mu \)g/mL PFDPA, PFBr, and chitosan (low molecular weight) separately in 10 mM PBS at 37 \(^\circ\)C. PFDPA and PFBr stock solutions were prepared initially in THF and vigorously added into the water with continuous shaking, and the final pellet was collected after centrifugation. These pellets were used further as inhibitors and incubated with A\( \beta \). Chitosan was dissolved in dilute acetic acid solution and diluted from this acidic solution to a final solution of 10 \( \mu \)g/mL in 10 mM PBS at pH 7.4. ThT fluorescence maintained almost a straight line profile in the presence of all the polymers unlike control (Figure 4b), suggesting a successful inhibition of amyloid aggregation. ThT profile also showed that in the presence of PC amyloid aggregation was nearly similar as in the case of control till the growth phase is reached. ThT intensity started decreasing after 60 h and followed a different path unlike control (Figure 4a, squares and circles). This divulged that unlike other precursors, PC was able to disturb selectively the more neurotoxic amyloid oligomers or other aggregation intermediates which existed in the growth phase and directed the amyloid aggregation in a different pathway.

**Changes in the Secondary Structure.** To investigate the changes in the peptide secondary structure in the presence of

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**Figure 2.** Cytotoxicity of PC2, PC3, PFBr, and PFDPA (0–100 \( \mu \)g/mL) in (a) human red blood cells and (b) endothelial cells (EA.hy926). Error bars correspond to standard deviations of six sets of experiments.

**Figure 3.** BBB assay of PC2, PC3, and PFBr (50 \( \mu \)g/mL). Optical images showing cellular integrity in the presence of (a) PC2, (c) PC3, and (e) PFBr during the experiment. Movement of (b) PC2, (d) PC3, and (f) PFBr through the endothelial monolayer from 0 to 4 h. Evans blue was used for control studies and the error bars correspond to the standard deviation of five experimental sets. The data points were fitted with Microsoft Excel second order polynomial for PC2 and PC3, whereas in PFBr, it is of third order.

**Table 1.** Permeability Efficiency of PFBr, PC3, and PC2 across the Endothelial Monolayer (BBB Permeability)

| time (h) | PC2 (%) | PC2 (\( \mu \)g/mL) | PC3 (%) | PC3 (\( \mu \)g/mL) | PFBr (%) | PFBr (\( \mu \)g/mL) |
|---------|---------|----------------|---------|----------------|---------|----------------|
| 0       | 0.0 ± 1.7 | 0.0 ± 0.9 | 0.0 ± 1.2 | 0.0 ± 0.6 | 0.0 ± 0.1 | 0.0 ± 0.0 |
| 1       | 46.9 ± 0.1 | 23.5 ± 0.0 | 18.1 ± 3.8 | 9.1 ± 1.9 | 0.2 ± 0.7 | 0.1 ± 0.4 |
| 2       | 55.0 ± 1.0 | 27.5 ± 0.5 | 28.4 ± 0.2 | 14.2 ± 0.1 | 5.2 ± 0.2 | 2.6 ± 0.1 |
| 3       | 53.8 ± 1.1 | 26.9 ± 0.5 | 41.5 ± 0.5 | 20.8 ± 0.2 | 10.0 ± 0.4 | 5.0 ± 0.2 |
| 4       | 45.9 ± 1.9 | 22.9 ± 0.9 | 44.1 ± 0.7 | 22.1 ± 0.4 | 9.1 ± 0.3 | 4.6 ± 0.1 |
| 6       | 37.5 ± 0.1 | 18.8 ± 0.1 | 6.5 ± 1.1 | 3.3 ± 0.6 | 0.1 ± 1.0 | 0.1 ± 0.5 |

**Figure 4.** ThT assay of only A\( \beta \)1–40 (20 \( \mu \)M, squares) and (a) PC (1 \( \mu \)g/mL) with coincubated A\( \beta \) oligomers (circles), A\( \beta \) fibrils (triangles), and CSF oligomers (15 \( \mu \)M, diamonds); (b) PFDPA (1 \( \mu \)g/mL, half-filled circles), PFBr (1 \( \mu \)g/mL, half-filled diamonds), and chitosan (low molecular weight, 1 \( \mu \)g/mL, half-filled triangles) coincubated with A\( \beta \)1–40 (20 \( \mu \)M) in 5 mM PBS (pH 7.4).
PC, the CD spectrum of only Aβ1–40, CSF (controls) as well as coincubated with polymeric conjugate were recorded at different time intervals.

All the samples were incubated at 37 °C in 10 mM PBS and diluted in 1 mM PBS before recording CD spectra. The spectra of only Aβ showed negative minimum (around 200 nm) upon 24 h incubation, confirming the formation of ordered β-sheet aggregation from the initial random coil. Small positive maxima at 191–193 nm and a negative minimum were observed in the control (Figure 5a, black line) Aβ1–40 solution. Aβ1–40 oligomers showed no such negative peak in the presence of PC. After 84 h incubation, the negative hump was reversed and a new positive maximum (around 196 nm) was observed (Figure 5a, red line).

This observation confirmed that PC successfully modulated Aβ oligomers and directed toward a different aggregation pathway as no increase in β-sheet conformation was found, unlike controls. Even in the case of preformed Aβ40 (20 μM) coincubated with PC (1 μg/mL), a small positive hump at 205 nm along with a negative peak at 194 nm appeared (Figure 5a, blue line). Ordered β-sheet aggregation of Aβ1–40 required for final fibril formation was proved to be disturbed in the presence of polymer conjugate PC and followed a different pathway. Interestingly, in the case of human CSF (15 μM) coincubated with PC (1 μg/mL) we observed a broad negative minimum from 210 to 230 nm and a positive maximum at 191–194 nm. Unlike as observed for the commercial Aβ1–40 oligomers, where PC was able to enhance the total fraction of β-turn and random coil formation, α-helicity was restored in the case of real CSF sample (Figure 5a, green line). Individual time-dependent CD spectra of Aβ1–40 oligomer, prefibrillar aggregates, and CSF were given in Supporting Information (Figure S1).

Further, to confirm the peptide secondary β-sheet formation, we have recorded FT-IR spectra of both controls and coincubated with polymeric conjugate (Figure Sb,c). In the case of controls, Aβ1–40 and CSF showed a peak at 1633 cm⁻¹ which indicated the presence of β-sheet conformation (Figure Sb). As seen from CD studies, the ordered peptide aggregation was disturbed in the presence of PC, as seen in FT-IR amide band at 1640 cm⁻¹ after 84 h incubation (Figure Sc). Most likely, β-rich oligomers were trapped in the presence of polymeric conjugate PC and due to the increased fraction of turn as observed in CD spectra, amide β-band appeared in FT-IR spectra in the presence of polymer conjugate. Further, these changes (modulation) were visualized and confirmed using AFM images.

**Morphological Changes.** Changes in amyloid aggregation in the presence of PC and other polymer precursors were studied using AFM. Aβ1–40 and CSF oligomers showed a similar AFM profile (Figure 6a–c). Aβ oligomers (Figure 6a) were 150 nm in diameter and 25 nm in height, whereas CSF oligomers (Figure 6c) were approximately 300 nm in diameter with a similar height profile as that of commercial Aβ1–40 oligomers (Figure S2). Preformed Aβ1–40 oligomers were incubated with PC at 37 °C, and after 84 h, larger aggregates (Figure 6b) of diameter 0.6 μm and 50–60 nm height (Figure S2) were seen to have developed. PC was able to block the oligomers from coming closer to each other and thus directed the peptide aggregation in a different pathway, inhibiting the final fibril formation unlike in the controls (Figure S3). Mature fibrils of Aβ1–40 and CSF showed a diameter of 150 nm and the average height was 6–9 nm. Further, to investigate the structural contribution of other polymer conjugate precursors on amyloid aggregation, Aβ1–40 oligomers were incubated separately with PFBr, PFDDA, and chitosan (1 μg/mL) and the changes in amyloid aggregation were captured after 84 h incubation at 37 °C (Figure S4). For the polyfluorene derivatives coincubated with Aβ1–40 oligomers, larger aggregates of 0.6–0.8 μm in diameter and 70 nm in height...
resulted. Peptide oligomers were trapped and no fibrillation had occurred. However, in the presence of chitosan coincubated with peptide oligomers, the sparse population of Aβ1–40 oligomers were visible along with chitosan co-aggregates showing particles of two different diameters and height profiles. Aβ1–40 oligomers showed a diameter of 100–150 and 20 nm in height as observed in controls and co-aggregates were found to have a larger diameter of 300 and 70 nm in height. Morphological observations suggested that a synergistic effect resulted in successful inhibition of amyloid fibrillation of toxic oligomers in Aβ1–40 and in CSF in the presence of PC. A similar surface motif of PC helped to adsorb the peptide oligomers to the polymeric surface and these hydrophobic nanoparticles dispersed in water would be able to modulate amyloid oligomers in Aβ1–40 and CSF to form mature fibrils.

**Optical Correlation.** Interestingly, PC was able to optically distinguish oligomers, unlike PFDPA. PFDPA orange emission in buffer was blue-shifted to 434 nm in the presence of Aβ1–40 aggregates (both monomers and prefibrillar aggregates) because of the formation of larger polyfluorene aggregates (as visible in AFM images, Figure S4a,b). However, the polymeric conjugate PC showed distinct optical features in the presence of oligomers. To figure out the polymer–protein interaction, Aβ1–40 (20 μM) and CSF (15 μM) oligomers were incubated in the presence of PC (2 mg/mL), and the optical spectrum of the coincubated samples were recorded. PC coincubated with Aβ1–40 oligomers showed an initial fluorescence emission at 570 nm (Figure 7a). Further incubation resulted in an increase in the fluorescence intensity with a blue shift of approximately 140 nm (Figure 7a, 429 nm) after 24 h incubation. Finally, a stable peak at 442 nm with a small hump at 543 nm appeared after 84 h incubation (Figure S5a). Thus, because of the similar surface, peptide oligomers were adsorbed onto the PC surface and the hydrophobic polymeric conjugate was able to interact with peptide oligomers. Finally, a stable polymeric aggregate was formed in the presence of peptide oligomers. A similar outcome also appeared in the presence of CSF oligomers (Figure 7b). An initial peak at 570 nm was blue-shifted at 436 nm after the immediate addition of CSF oligomers to the polymeric solution. The response found in CSF was weaker compared to commercial Aβ1–40 because of lesser concentration of Aβ in CSF and the presence of preformed oligomers (immediate response was better compared to Aβ1–40 coincubated sample). Finally, in CSF coincubated with polymeric solution, a stable peak at 442 nm and a small hump at 547 nm resulted after 84 h incubation as it was observed in case of the Aβ1–40 coincubated sample (Figure S5b). To rule out the contribution of polymeric aggregation, we have incubated pristine polymer solution in 10 mM PBS buffer at 37 °C. No blue shift was observed in the absence of peptide supporting the polymer–protein interaction. From these optical changes, we confirmed the selectivity of PC on Aβ1–40 oligomers over other precursor’s viz., PFBr, PFDPA, and chitosan. The selectivity studies were also performed, and the fluorescence spectra of PC coincubated with neurotoxic brain metals and other amino acids up to 100 μM were recorded. A decrease in the initial polymeric peak at 570 nm was observed, but in no case blue shift was found as observed in the presence of oligomers (Figure S6). Mutual aggregation resulted in a stable optical response because of the probable interaction of polymer conjugates and early amyloid aggregates or oligomers and formation of distinct polymeric aggregates, which was highlighted in the optical profile as a hypsochromic shift.

### DISCUSSION

Crossing the BBB is one of the chief hurdles to deliver potential therapeutic or diagnostic molecules to the brain. It is known that there are approximately 100 billion capillaries and a BBB surface area of 20 m² in the human brain, as compared to much smaller 0.021 m² for the blood–cerebral spinal fluid barrier which controls entry of molecules via this barrier. To prescribe a successful modulator, one must address the two most important factors, viz., targeting robust and heterogeneous amyloid aggregates and the ability to cross BBB. Conjugate polymer nanoparticles have previously been reported as modulators of amyloid aggregates. In the present study, we have modified the conjugate polymer (polyfluorene, PFDPA) nanoparticle with chitosan and utilized the polymeric composite (PC) as a modulator. The primary thrust for the development of nanoparticles is the hydrophobic effect as the polymer chains have a tendency to abstain from water and therefore fold into round shapes. Additionally, these polymer nanoparticles did not interact with free metals and other amino acids. ThT assay (Figure 4) and fluorescence studies (Figure 7) inferred that these nanoparticles can be directed to target selectively Aβ oligomers. Further, distinct optical response of PC in the presence of Aβ1–40 aggregates (Figure 7b) confirmed the formation of stable polymer–peptide co-aggregates. The formation of these co-aggregates was confirmed by morphological studies (Figure 6b,d), and CD spectra showed no parallel β-sheet formation in the peptide secondary structures (Figure 5a). Weak amide stretching shifted to 1640 cm⁻¹ from 1633 cm⁻¹ in the presence of PC inferred the coexistence of both polymer and protein oligomers but directed amyloid aggregation into a nonfibrillar pathway as evident from AFM images. In oligomers, the hydrophobic core is placed deep inside, and therefore, they are more robust to modulate. A similar structural motif (Figure 1) helped them to adsorb into the polymer surface, and thus, the proximity to other oligomers is prohibited (Figure 6b) and thus modulated final fibril formation. Nevertheless, several other issues remain to be checked, which includes the essential function of BBB through influx and efflux in the presence of PC and how the brain plasticity changes after in vivo experiments, but these are presently beyond the existing scope. Finally, a new structural motif has been presented with an idea of how to prevent or attack early amyloid aggregates in order to prevent presenile

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![Figure 7](image-url)
dementia. This will definitely benefit to understand the early aggregation and develop biomarkers to slow neurodegeneration.

**CONCLUSIONS**

In summary, this new polymer conjugate (polyfluorene–chitosan, PC) was able to optically sense oligomers as well as modulate the toxic amyloid aggregates in Aβ1–40 and in CSF. ThT assay showed a response in the growth phase targeting the pre fibrillar aggregates which were further validated by fluorescence emission of the polymer conjugate in the presence of in vitro Aβ1–40 and CSF oligomers. Precursor polyfluorene derivatives were also shown to inhibit amyloid aggregation as well, indicating a synergistic effect played an important role in modulating amyloid oligomers. CD spectra showed no parallel β-sheet formation which is considered as the pathological progression of amyloid aggregation and results in fibril formation and has been modulated by the polymer conjugate. PC was also able to cross BBB efficiently irrespective of the molecular weight of the additive without disintegrating the endothelial monolayer, unlike polyfluorene precursors. Hydrophobic conjugate nanoparticles can be made efficient amyloid modulators, which not only can cross BBB efficiently but also can target selectively the early neurotoxic amyloid oligomers and modulate these robust aggregates into a nonpathological pathway. We believe that our findings may open a new hope of making therapeutic nanomaterials that can be used to predict and fight against Alzheimer’s and presenile dementia.

**EXPERIMENTAL PROCEDURES**

**Materials.** Chitosan low molecular weight (PC2) and high molecular weight (PC3), di-(2-picolyl)amine, 1,6-dibromohexane, fluorene, ferric chloride, potassium carbonate, PBS, HFIP, TFA, Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), and other chemicals were obtained from Merck, Sigma-Aldrich Chemicals, Ranbaxy (India) and used as received. GL Biochem Ltd., Shanghai, China supplied the human amylloid-β (1–40). CSF and human samples were collected from Guwahati Neurological Research Center and Hospital, Six Mile, Guwahati, India.

**Synthesis of PFDPA and PC.** Fluorene molecules were first alkylated using 1,6-dibromohexane in 50% aqueous sodium hydroxide, and the crude was purified using column chromatography, which was done using eluent hexane to obtain the desired doubly alkylated product as a viscous yellow liquid. Then, the purified double alkylated monomer was polymerized via oxidative polymerization using ferric chloride as a catalyst and nitrobenzene as a solvent under an argon atmosphere. The resulting polymer was precipitated in methanol and dried under vacuum, and finally, to prepare PFDPA, appended bromines were substituted by di-(2-picolyl)amine in dimethylformamide and K2CO3. The desired polymer PFDPA was purified via precipitation from methanol, and the attachment was confirmed via 1H and 13C NMR spectra in chloroform-d (Scheme S1). For preparing the nanoconjugates (PFDPA–chitosan, PC), chitosan (100 µg of 1 mg/mL) was added in 1 mL of PFDPA (1 mg/mL) in an Eppendorf (vortex condition) for 30 min. The orange-brown intense PC nanoconjugate solution was ultracentrifuged (14 000 rpm at 4 °C for 30 min). The PC nanoconjugate pellet (50 µL) was utilized for all the studies. PFDPA was also centrifuged under similar conditions, and the pellet was used to compare with PC (Scheme S1) for all experiments.

**Instrumentation.** AFM images were taken using Bruker, Innova, with noncontact tapping mode using a large scanner. Malvern Zetasizer Nano series Nano-ZS90 instrument was used to measure dynamic light scattering. CD measurements were done in a JASCO J-1500-150 spectrometer (JASCO Co. Tokyo, Japan), using a quartz cuvette (1 mm path length). NMR spectra of PFBr, PFDPA, and precursor monomers were taken in a Bruker Ascend 600 MHz spectrometer using CDCl3 as a solvent. ThT fluorescence measurements were done in a Tecan microplate reader using Corning 96 flat bottom black, clear bottom polystyrene cat. no. 3631 plate reader. The fluorescence spectra was recorded in 1 mL solution, with a 10 mm x 10 mm quartz cuvette, collecting the emission at 90° relative to the excitation beam using FluoroMax-4 spectrofluorometer-Horiba Scientific.

**In Vitro Cell Viability and BBB Assay.** To check the intrinsic toxicity of all the precursor polymers (PFBr, PFDPA) along with the final conjugate (PC), in vitro toxicity was studied by both hemolysis assay and the MTT cell survival assay with RBCs and Ea hy926.1, respectively, as described in our previous report. Endothelial cells (EA hy926.1) were harvested in DMEM (HiMedia) with 10% FBS (Gibco) and antibiotics (Anti–Anti, Gibco) at 37 °C in a 5% CO2 incubator, and 25 000 cells were seeded per well in a 96-well plate. The cells were treated with different concentrations of PFBr, PFDPA, and PCs (0–100 µg/mL) for 12 h, and the cell survival was determined by standard MTT assay. Prior to cell viability, in vitro BBB assay was performed by making an endothelial monolayer barrier. Briefly, endothelial cells were seeded in a special cell culture plate (60 mm), which possesses 3 µm pores for crossing. Endothelial cells were grown densely till completely sealing the pores. This plate was maintained in a 100 mm sterile plate with an adequate amount of media. PFBr, PFDPA, and PC solutions (50 µg/mL) were prepared in complete media and gently added in three different 60 mm dishes (upper chamber), and the media was collected from the 100 mm dish (lower chamber) after every 1 h interval till 6 h. Further, the permeability was checked by measuring fluorescence of all the test compounds. Leakage was corrected by using Evans blue as a control and to calculate the actual permeability of the precursor polymers and conjugates.

**Preparation of Stock Solution.** PFDPA and PFBr stock solutions (1 mM) were prepared in 10 mL of THF and diluted to necessary concentrations for further incubation during modulation and imaging studies. PC conjugates were prepared at a concentration 20 mg/mL in 10 mM deionized water, and finally, 1 mg/mL was diluted in PBS for modulation studies. All the experiments such as UV−visible, FT-IR and fluorescence titrations were performed in 10 mM PBS buffer and pH maintained at 7.4. PFDPA (10 µM) was injected into deionized water at regular intervals with vigorous stirring at room temperature and filtered via a membrane filter with 0.2 µm pore size and used for other studies. Chitosan was dissolved in 2% acetic acid solution and the conjugates were freshly prepared in deionized water.

**Oligomerization of Aβ(1–40).** TFA/HFIP was used to disaggregate Aβ(1–40) initially following an earlier protocol to obtain a final concentration of 0.2 mM. Then, it was kept at 37 °C for 24 h in dark conditions for oligomerization and confirmed by recording AFM images using a Bruker, Innova instrument. Further fibril formation and modulation were
examined with ThT fluorescence assay in a Tecan microplate reader.

Preparation of Aβ1–40 Aggregates and ThT Binding Assay. Aβ1–40 and CSF were used as a source of amyloid oligomers. Aggregation of Aβ1–40 (20 μM) was examined by incubating with ThT (40 μM) at 37 °C for 24 h (pH 7.4 in 10 mM PBS) without stirring to prepare oligomeric amyloid peptide aggregates. Further, aggregation of Aβ1–40 amyloid fibrils were checked both in the presence and absence of modulators with different time incubations by regularly monitoring ThT (40 μM) fluorescent enhancement peak at λex 482 nm (λem 440 nm) using a microplate reader. Similarly to confirm the presence of Aβ aggregates in human CSF sample, first ThT fluorescence was measured in the saline buffer and corrected with Aβ1–40 reading as a control. AFM images validated the presence of CSF oligomers, which similarly formed mature fibrils on further incubation in saline buffer.

Modulating Experiment for Aβ1–40 Aggregates. The ability of the polymeric conjugate (PC) to modulate oligomers was studied by recording the changes in the fluorescence spectra in the presence of Aβ1–40 in commercial and in real CSF samples as well as studying CD spectra to investigate the changes in the secondary structure. The samples were prepared in the final volume of 1 mL in 10 mM PBS (pH 7.4) buffer and incubated at 37 °C with Aβ1–40 and CSF separately. Fluorescence spectra were recorded at a different time intervals to correlate protein aggregation and the optical signal of the polymeric conjugate. First, when Aβ1–40 oligomers were incubated with PC (2 mg/mL) solution and excited at polymeric conjugate excitation (364 nm), we observed an increment in the secondary structure. The samples were prepared to incubated in PC (2 mg/mL) solution and excited at polymeric conjugate excitation (364 nm), and fluorescence was measured in the saline buffer and corrected with Aβ1–40 reading as a control. AFM images validated the presence of CSF oligomers, which similarly formed mature fibrils on further incubation in saline buffer.

## ASSOCIATED CONTENT

> Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00764.

- Synthesis of PFDPDA, CD, and AFM of peptide aggregates both in the presence and absence of PC, and photoluminescence studies of PC-containing relevant amyloid aggregates, neurotoxic metal ions, and amino acids (PDF)

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**Notes**

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## ABBREVIATIONS

CSF, cerebrospinal fluid; Aβ, amyloid β; CD, circular dichroism; AFM, atomic force microscopy; PC, polymer conjugate; BBB, blood–brain barrier; ThT, thioflavin T

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