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Cationic polymethacrylate-copolymer acts as an agonist for β-amyloid and antagonist for amylin fibrillation

Bikash R. Sahoo*, Takuya Genjo*, Takahiro W. Nakayama, Andrea K. Stoddard, Toshio Ando, Kazuma Yasuhara, Carol A. Fierke, Ayyalusamy Ramamoorthy

In human, β-amyloid and islet amyloid polypeptide (IAPP, also known as amylin) aggregations are linked to Alzheimer’s disease and type-2 Diabetes, respectively. There is significant interest in better understanding the aggregation process by using chemical tools. Here, we show the ability of a cationic polymethacrylate-copolymer (PMAQA) to quickly induce β-hairpin structure and accelerate the formation of amorphous aggregates of β-amyloid-1-40, whereas it constrains the conformational plasticity of amylin for several days and slow down its aggregation at substoichiometric polymer concentrations. NMR experiments and microsecond scale atomistic molecular dynamics simulations reveal that PMAQA interacts with β-amyloid-1-40’s residues spanning regions K16-V24 and A30-V40 followed by β-sheet induction. For amylin, it binds strongly to the closest proximity of the amyloid core domain (NFGAIL) and restrains its structural rearrangement. High-speed atomic force microscopy and transmission electron microscopy experiments show that PMAQA blocks the nucleation and fibrillation of amylin, whereas it induces the formation of amorphous aggregates of β-amyloid-1-40. Thus, the reported study provides a valuable approach to develop polymer-based amyloid inhibitors to suppress the formation of toxic intermediates of β-amyloid-1-40 and amylin.

Introduction

Self-assembly of amyloidogenic proteins is involved in numerous neurodegenerative diseases and other disease including Alzheimer’s disease (AD) and Type-2 Diabetes (T2D). Nevertheless, our current understanding of the role of protein aggregation in the pathogenesis of such diseases remains elusive. Despite the recent advancements in high-throughput screening of several anti-amyloidogenic compounds, there is no treatment for the protein aggregation based disorders including AD and T2D. β-amyloid and islet amyloid polypeptide (amylin) aggregations are linked to AD and T2D, respectively. Both β-amyloid and amylin follow a conserved aggregation pathway and form toxic oligomer intermediates. Thus, it has been the subject of intense research to establish a pathological correlation and develop small molecules that interfere with the aggregation pathways of β-amyloid and amylin. The sequential protein aggregation mechanism from a water-soluble monomer to insoluble amyloid fibers triggering AD and T2D still remains elusive. However, several studies have observed a conserved pathway where both β-amyloid and amylin monomers aggregate to form pre-fibrillar toxic oligomers followed by mature β-sheet rich fiber structures. Thus, the sequential aggregation products of β-amyloid or amylin have been targeted to design potential inhibitors to interrupt amyloid formation. Moreover, substantial research effort has been devoted in developing strategies to reduce the formation of toxic intermediates of β-amyloid or amylin. In response to this, several amyloid inhibitors or modulators have been clinically tested for AD or T2D treatment. However, small molecule inhibitors or modulators targeting amyloidosis have recently faced several clinical trial challenges. In addition, the complexities associated with amyloid aggregation such as biological pathways, involvement of other cellular key players, structural heterogeneity etc. pose challenges to current therapeutic developments. Among several compounds, the chemically conserved scaffold molecules characterized by multi aromatic groups have been tested in vitro or in vivo. These compounds have been reported to modulate the amyloid aggregation pathways. But the poor solubility and bioavailability affect their therapeutic nature and recently have been shown to be overcome using nanocarriers. Although, their mechanism of action remains unknown, covalent bonding, hydrogen bonding, hydrophobic interactions and π-π stacking are thought to be the major driving forces for their inhibitory mechanism of action. Oxidation of amyloid peptides by chemical compounds can also modulate amyloid aggregation. Despite substantial efforts, no significant new drugs have been discovered against these most tenacious and unnerving medical disorders.
direct researchers to develop new anti-amyloidogenic molecules such as polymers, peptoids, macrocyclic peptides, nanoparticles, molecular chaperones etc. Among them, several polymers characterized by their ionic properties have been tested to investigate their activities on amyloidogenic aggregation. Notably, amine containing polymers, polyamino acids, cationic surfactants and cellular polyamines have been observed to modulate β-amyloid aggregation. Similarly, controlled aggregation kinetics and toxicity of amylin using star-polymers and β-amyloid using polymer-nanodiscs have been studied recently. Here we demonstrate the modulation of amyloid aggregation pathways for amylin and β-amyloid-1-40 using a polyacrylamide derived copolymer (PMAQA) that has been implicated in several biological studies including lipid-nanodiscs formation, enhancement of drug-delivery, modulating bioavailability and microencapsulation.

Experimental section

Materials

The polymethacrylate quaternary ammonium copolymer (PMAQA, ~1.7 kDa) was synthesized and purified as reported elsewhere. Unlabeled and uniformly 15N isotope labeled full-length β-amyloid-1-40 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVQ) was recombinantly expressed in E. coli BL21 (DE3). The β-amyloid-1-40 expression was followed from previously described protocols and purified by loading the samples to an ECOPLUS HPLC column packed with the reversed-phase separation material. Synthetic human amylin (KNCATCATCAQLANFVHSSNNGAILSSTVGNSNTY-NH2) was purchased from AnaSpec at >95% purity.

Sample preparation

The sample preparation was followed from the previously described method. Briefly, 1 mg/mL β-amyloid-1-40 peptide was dissolved in 5% (v/v) NH4OH and aliquoted to 0.1 mg/mL followed by lyophilization. The β-amyloid-1-40 peptide powder was re-suspended in 10 mM sodium phosphate, pH 7.4 and sonicated for 30 s followed by centrifugation at 14,000 × g for 15 min at 4 °C to remove small aggregates. 1 mg/mL amylin was treated with 1,1,1,3,3,3-hexafluoropropanol (HFIP) and kept on ice for 30 minutes. The peptide solutions were aliquoted to 0.1 mg/mL and lyophilized. The amylin powder was re-suspended in 30 mM sodium acetate buffer, pH 5.5 and sonicated for 30 minutes. The peptide solutions were aliquoted to 0.1 mg/mL and purified by loading the samples to an ECOPLUS HPLC column packed with the reversed-phase separation material. The resultant solutions were purified using size exclusion chromatography (SEC) by passing through a superdex 200 Increase 300/10 GL column operated on an AKTA purifier (GE Healthcare, Freiburg, Germany).

NMR

1D and 2D NMR spectra were recorded on a 600 MHz Bruker Avance III NMR spectrometer equipped with a z-axis gradient cryogenic probe. Unlabeled amylin (50 μM) dissolved in 30 mM sodium acetate, pH 5.5, and unlabeled β-amyloid-1-40 or uniformly 15N-labeled β-amyloid-1-40 peptides (60 μM) dissolved in 10 mM sodium phosphate, pH 7.4, buffer containing 90% H2O/10% D2O was used for NMR measurements. The 2D 1H/1H SOFAST-HMQC NMR titration experiments of β-amyloid-1-40 (60 μM) with 0.6, 1.2 and 3 μM PMAQA were recorded at 10 °C with 64 scans and 200 t increments. The NMR spectra were processed using TopSpin 3.5 (Bruker) and analyzed using Sparky.

High-speed AFM and Transmission Electron Microscopy imaging

A high-speed atomic force microscopy (HS-AFM) instrument was operated at the tapping mode with a small cantilever (BL-AC10DS-A2, Olympus with spring constant k = 0.1 N/m, and resonance frequency f = ~400 kHz in water). Each cantilever has an amorphous carbon tip at its top. The tip was prepared by electron beam deposition with a scanning electron microscope (EIS-7500, Elionix). The free oscillation amplitude of the cantilever and the typical set-point were ~1 nm and 80% of the free oscillation amplitude. For the HS-AFM sample stage, a freshly cleaved mica disc (of 1 mm in diameter) fixed on a glass rod (of 2 mm in diameter) was used. Before the deposition of amylin fibril seed, each mica disc was freshly cleaved. 3-aminopropyltriethoxysilane (APTES)-modification, mica was immersed with 2 µL of ten-thousand-fold dilution of APTES with water followed by the removal of unbound APTES with 60 µL of water. Amylin fibril seeds for the imaging were prepared by incubation of 60-100 µL of 5 µM amylin in 30 mM sodium acetate, pH 5.5 for 2-3 days followed by sonication with a handheld sonicator (UR-21P, Tomy). Validation of the sonication condition was checked from AFM images (typical sonication condition: ten times of ~0.5 s at the output level 3). 2 µL of fibril seeds was deposited on bare mica or APTES-modified mica followed by incubation for about 5 minutes. After the removal of unbound seeds with 20 µL of 30 mM sodium acetate, pH 5.5, the scanner with the sample stage was set on the scanner holder so that the stage was immersed in 60 µL of 30 mM sodium acetate, pH 5.5, in the sample chamber with the cantilever. After the observation of the fibril seeds, the buffer solution in the chamber was replaced...
with 5 μM amylin with/without equimolar PMAQA. HS-AFM observation was started just after the replacement of the sample chamber solution. The HS-AFM movie analysis and kymographs were prepared using ImageJ (NIH) plugin as described elsewhere.40

5 μM of β-amyloid-1-40 monomers dissolved in 10 mM sodium phosphate, pH 7.4 or 5 μM of amylin monomers dissolved in 30 mM sodium acetate, pH 5.5, were incubated for 24 hours under continuous agitation with/without equimolar concentration of PMAQA. β-amyloid-1-40 or amylin fibers were prepared by incubating 5 μM of peptide monomers in respective buffer as described above at room temperature for 3 days under continuous agitation. Then, the fibers were sonicated using an ultrasonic bath sonicator for 2 hours at room temperature to generate short fibers, which were used as amyloid seeds. The sonicated fibers were incubated with 5 μM of PMAQA in respective peptide buffers at room temperature for 24 hours under continuous agitation to check the effect of polymer on fiber morphology. In addition, to monitor PMAQA’s effect on the self-seeding reaction, sample solutions containing sonicated fibers were incubated with 5 μM monomers of respective peptides for 24 hours in the presence or absence of 5 μM PMAQA. 10 μL of all sample solutions described above were added to a collodion-coated copper grid and incubated for 3 minutes at room temperature followed by three times rinsing with double deionized water. Then, the copper grid was stained with 3 μL of 2% (w/w) uranyl acetate and incubated for 2 minutes followed by three times rinsing with double deionized water. The sample grids were dried overnight under vacuum and used to obtain transmission electron microscopy (TEM) images using a HITACHI H-7650 transmission microscope (Hitachi, Tokyo, Japan) at 25 °C.

**MD simulations**

The 2D structure of PMAQA (Fig. S1) was created using ChemDraw 16.0 and exported to Chem3D for energy minimization. The 3D structure of PMAQA and its topology files were created using ATB builder41 from the energy minimized 2D structure for all-atom molecular dynamics (MD) simulation. The solution NMR structures of β-amyloid-1-4042 (PDB ID: 2LFM) and amylin43 (PDB ID: 5MGQ) were considered as the initial structures for PMAQA interaction analysis; the amino acid sequences of the peptides are shown in Fig. S1. The MD system was built in GROMACS44 software package, version 5.0.7 (GROMOS56 54A745 force field), by placing β-amyloid-1-40 or amylin at the center of a cubic box and the polymer ~1 nm away from the protein. The MD systems were solvated using SPC/E water (= 1000 kg m⁻³) and neutralized by adding counter ions followed by energy-minimization using the steepest-descent method. Short NVT (100 ps) followed by 1 ns NPT (310 K and 1 bar) was performed to equilibrate the MD systems. MD simulations were carried out using 3D periodic boundary conditions over a production run of 0.7 and 1μs for amylin-PMAQA and β-amyloid-PMAQA systems, respectively. MD trajectories were interpreted using visual molecular dynamics46 and images were built using Discovery studio visualizer 3.5. The binding free energy was calculated for amylin-PMAQA and β-amyloid-PMAQA complexes using MM/PBSA by retrieving 500 structures from the last 100 ns MD simulation.47 The GMXAPBS tool was used for the free energy calculation as described elsewhere.48-50

**Results and discussion**

The effect of PMAQA on β-amyloid-1-40 and amylin aggregation kinetics was monitored using ThT based fluorescence assay. ThT aggregation assays monitored for 4 days showed opposite activities for β-amyloid-1-40 and amylin aggregation in the presence of PMAQA (Figs. 1 and S2). As shown in Fig. 1 (a and b), PMAQA accelerated β-amyloid-1-40 and slowed down amylin aggregations. At a low polymer concentration, i.e. peptide:PMAQA molar ratio of 1:0.02 or 1:0.04, a significant difference in the lag-times of β-amyloid-1-40 and amylin aggregation was ascertained (Fig. 1a and b). In the presence of 0.02 μM of PMAQA, the β-amyloid-1-40 fibrillation growth was observed after two days (blue) as compared to the aggregation after three days for the control sample (red). A slight increase in the polymer concentration, i.e. 0.04 μM of PMAQA (green), significantly accelerated β-amyloid-1-40 fibrillation with an increased ThT fluorescence intensity (Figs. 1a and S2a). While further increase in the polymer concentration to 1.02 or 1.05 β-amyloid:PMAQA molar ratio also accelerated the peptide aggregation. But, a relatively lower ThT intensity was observed for 1:0.5 as compared to that obtained for 1:0.2 or 1:0.04. As shown in Fig. 1a, the observed ThT intensities are higher for all the substoichiometric concentrations of PMAQA than the control (i.e., in the absence of PMAQA). At equimolar or superstoichiometric concentration of PMAQA (Figs. 1a and S2b), β-amyloid-1-40 fibrillation was ~6 times faster than that observed for the control sample.

Unlike the effect of PMAQA on β-amyloid-1-40 fibrillation, amylin aggregation was slowed down at substoichiometric PMAQA concentration, i.e. amylin:PMAQA molar ratio of 1:0.02 or 1:0.04 (Fig. S2c). Increasing the polymer concentration to 1:0.2 amylin:PMAQA molar ratio further slowed down the aggregation with a lag-time over 2 days as compared to its aggregation in ~1 day in the absence of PMAQA (Fig. 1b). Further increase in PMAQA concentration, i.e. at equimolar polymer:peptide or superstoichiometric PMAQA concentrations, amylin aggregation was significantly slowed down as revealed from the negligibly small ThT fluorescence intensity (Figs. 1b and S2d). The observed increase in the ThT intensity in both peptides at substoichiometric PMAQA concentrations (peptide:polymer 1:0.5 or lower) indicates the presence of a high quantity of β-amyloid or amylin fibers. On the other hand, at superstoichiometric PMAQA concentrations (peptide:polymer 1:1 or higher), a substantial decrease in the ThT intensity indicates the presence of less amount of fibers. Specifically, the low ThT intensity observed for β-amyloid-1-40 could also indicate the formation of...
morphologically distinct fibers, i.e. amorphous like aggregates that depict low ThT fluorescence intensity.51

A partial helical CD spectrum containing 9.4/26.1% of α/β secondary contents (as estimated by BESTSEL 52) was observed at 1:0.2 β-

Table 1 Secondary structure assessment (%) of amylin from CD spectra (Fig. 2d and f) in the absence or presence of PMAQA by BESTSEL.52

<table>
<thead>
<tr>
<th>Time</th>
<th>0 d</th>
<th>1 d</th>
<th>2 d</th>
<th>3 d</th>
<th>4 d</th>
<th>5 d</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amylin in 30 mM sodium acetate, pH 5.5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-helix</td>
<td>0.7</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>βαβ</td>
<td>42.1</td>
<td>37.5</td>
<td>54.0</td>
<td>50.9</td>
<td>53.1</td>
<td>53.0</td>
</tr>
<tr>
<td>βαν</td>
<td>0</td>
<td>3.9</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
</tr>
</tbody>
</table>

| **Amylin+PMAQA in 30 mM sodium acetate, pH 5.5** |     |     |     |     |     |     |
| α-helix | 14.5 | 9.1 | 10.6 | 5.5 | 5.3 | 6.6 |
| βαβ   | 21.7 | 29.7 | 28.1 | 30.3 | 33.4 | 25.4 |
| βαν   | 0   | 0   | 5.7 | 3.7 | 13.4 |

We next monitored the stability of the PMAQA induced β-

amyloid-1-40 or amylin secondary structures for 5 days by incubating the peptides at room temperature in the absence or presence of 1.5 molar equivalents of PMAQA (Fig. 2c-f). The time-lapse CD spectra of β-amyloid-1-40 or amylin in the absence of PMAQA showed a sequential structural transition from a random-coil rich (negative peak at 200 nm) to β-sheet structure (Fig. 2c and d). While β-amyloid-1-40 in solution are reported to exhibit a sequential structural change to form cross β-sheet structures over several days (Fig. 2c),53 in the presence of 1.5 molar equivalents of PMAQA a rapid β-sheet induction within several minutes was observed (Fig. 2e). The observed rapid structural change correlates with the ThT aggregation kinetics (Fig.1a) and indicates a reduction in the level of potential β-

amyloid-1-40 oligomers that are reported to be neurotoxic.53,54

Similar to the amyloidogenic property of β-amyloid-1-40, amylin in solution after 2 days depicted a CD spectrum with a maximum at ≈200 and a minimum at ≈218 nm indicating its transitory state characterized by an increasing percentage of β-sheet (54%) (Fig. 2d). But, unlike β-amyloid-1-40, and as observed in ThT assays for amylin (Fig.1b), CD spectra showed a relatively slow change in amylin's secondary structure when incubated with 1.5 molar excess of PMAQA. The CD minimum was observed at ≈200 nm up to day-3 and the spectra were quantified using BESTSEL (Fig.2f, Table 1). Secondary structure assessment of PMAQA bound amylin from CD spectra showed a relative increase in α-helix (14.5%) and decrease in β-sheet (21.7%) content as compared to that observed in the absence of PMAQA (Table 1). In addition, an increased percentage of parallel β-sheets (13.4 % on day-5) was observed for amylin in the presence of PMAQA over time as compared to 0% (on day-5) in the absence of PMAQA. This observation indicates that PMAQA bound amylin could have both anti-parallel and parallel β-structures. Such observations have been found previously using X-ray crystallography in different segments of amylin (segment 13-18 with parallel and anti-parallel β-structures for segments 16-21, 22-28, and 23-29).55

On the other hand, β-amyloid-1-40 showing a positive at ≈200 and negative at ≈225 nm CD bands in the presence of PMAQA indicates the formation of a predominant β-sheet structure (Fig.2e). The observed CD minimum with a red-shift (from ≈222 on day-1 to ≈225
Next, we carried out FT-IR experiments on samples after 6 hours of incubation under similar experimental conditions used for CD measurements to further confirm the observations from CD experiments. As shown by the FT-IR spectra in Fig. 2g, predominant β-sheet structures of β-amyloid-1-40 were observed as indicated by a sharp amide I peak at 1628 cm⁻¹ along with an increasing percentage of turns (at 1675 cm⁻¹) in the presence of PMAQA, which are in qualitative agreement with CD results shown in Fig. 2e. In the case of amylin, FT-IR spectra showed a minor change in the characteristic of the helical band at 1650 cm⁻¹ in the presence of PMAQA (Fig. 2h). This indicates that PMAQA binding restraints the conformation of partially folded amylin. The β-sheet rich β-amyloid and partially folded amylin conformations in the presence of PMAQA (Fig. 2) coupled with ThT observations (Fig. 1) indicated the presence of distinct peptide species such as fibers or low-order aggregates. To probe this, SEC profiling was performed to quantify the size distribution of the PMAQA bound peptide species. SEC analysis of β-amyloid-1-40 incubated with 1.5 molar excess of PMAQA for ~5 minutes at room temperature showed two different elution profiles. The fractions collected at ~5 to 12 mL correspond to amyloid fibers or protofibers and that collected at ~20 to 25 mL are free polymers or low-order or monomeric β-amyloid-1-40 (Fig. 3a). Remarkably, SEC profile for PMAQA-amylin mixed solution, incubated for a relatively longer time (overnight) at room temperature, exhibited low-order or monomeric amylin and free polymers eluted at ~20 to 25 mL (Fig. 3b). Taken together, the above described experimental results present a counter active role of PMAQA on β-amyloid-1-40 and amylin aggregation.

Next, we studied the binding mechanism of PMAQA with β-amyloid-1-40 or amylin using an integrated NMR and MD simulation approach. The amide-NH region of 1H NMR spectra obtained in the absence of PMAQA showed a monomer or low-order aggregates for both β-amyloid-1-40 and amylin as indicated by the dispersed NMR peaks (Fig. 3). A substantial change in 1H NMR spectra of β-amyloid-1-40 was observed at 1.02 β-amyloid:PMAQA molar ratio (Fig. 3, blue trace). Remarkably, the loss of amide-1H peaks of β-amyloid-1-40 was observed when the PMAQA concentration (1.005 β-amyloid:PMAQA ratio) was slightly increased (Fig. 3d). This agrees with the PMAQA induced β-amyloid-1-40 aggregation observed in ThT assays. In contrast, the amide peaks of amylin were observed even when titrated with 1.2 molar equivalent of PMAQA (Fig. 3, blue trace). These NMR findings are in good agreement with the observed conformational transition from CD and ThT based aggregation results (Figs. 1b and 2b). Interestingly, a significant line broadening observed for the proton peak of PMAQA’s –NR³⁺ group (at 2.97 ppm) in the presence of β-amyloid-1-40 which indicates the interaction of –NR³⁺ with β-amyloid-1-40. In contrast, a sharp proton peak for PMAQA’s –NR³⁺ was observed in amylin solution (Fig. 3). This observation most likely indicates the formation of an electrostatic interaction between the cationic PMAQA and anionic β-amyloid-1-40, as β-amyloid-1-40 (at pH=7.4) and amylin (at pH=5.5) carry negative (-3) and positive (+4) charges, respectively. Thus, while the cationic group of PMAQA binds strongly to anionic β-amyloid-1-40, a repulsive force could be expected in the presence of cationic amylin.

To gain further atomistic insight into the mechanism, β-amyloid-1-40 aggregation was monitored using 2D 1H/15N SOFAST-HMQC experiment at variable substoichiometric polymer concentration; since 1H NMR of amylin showed very little change in the amide region at superstoichiometric PMAQA concentration, further NMR experiments were not carried out on amylin. As shown in Fig. 4a-d, intensity reduction and chemical shift changes were observed for β-amyloid-1-40 residues indicating a PMAQA induced structural rearrangement for β-amyloid-1-40. A substantial loss of signal intensity was observed in β-amyloid-1-40 spanning regions K16-V24 and A30-V40 (as per previously published assignments) indicates a potential site of PMAQA’s interaction with β-amyloid-1-40 at 1:0.01

Fig. 3 1H NMR spectra of β-amyloid-1-40 or amylin. NMR spectra showing the interaction of PMAQA with β-amyloid-1-40 (60 µM β-amyloid-1-40, 1.2 µM PMAQA dissolved in 10 mM sodium phosphate, pH 7.4) or amylin (50 µM amylin, 60 µM PMAQA dissolved in 30 mM sodium acetate, pH 5.5). The change in the NMR signal intensity of the –NR³⁺ proton in PMAQA or protein amide region (H-N) is highlighted.
and 1.002 β-amyloid:PMAQA molar ratio (Fig. 4b and c). A further increase in PMAQA concentration (1.005 β-amyloid:PMAQA molar ratio) increased the line-broadening and also resulted in the loss of 15N/1H resonances (Fig. 4d). This indicates that PMAQA interaction with β-amyloid-1-40 induces the aggregation of the peptide.

To further explore the binding mechanism of PMAQA with amylin or β-amyloid-1-40 at structural level, we performed all-atom MD simulation on a time scale of 0.7 or 1 µs, respectively. Structural insights into the PMAQA interaction with β-amyloid-1-40 (a) or amylin (b) shown as cartoon. The PMAQA binding peptide amino acids (shown in sticks) are labelled and hydrogen bonds are shown in black dashed lines in Discovery Studio Visualizer. (c) Peak intensities measured from 2D SOFAST-HMQC spectra (Figure 4) of 60 µM β-amyloid-1-40 in the absence or presence of 0.6 µM PMAQA. The yellow area highlights the β-amyloid-1-40’s residues with significantly reduced signal intensities. (d) Root mean square fluctuation (RMSF) of residues in β-amyloid-1-40 (grey) or amylin (red) interacting with PMAQA derived from 1 or 0.7 µs MD simulations, respectively. The yellow region indicates a comparatively flexible β-amyloid-1-40 N-terminal domain. The blue and orange regions indicate aggregation core domains of amylin and β-amyloid-1-40, respectively. The average RMSF value is shown using dashed horizontal lines.

### Table 2. MM/PSA based binding free energy (kcal mol⁻¹) calculation.

<table>
<thead>
<tr>
<th>Polar Contribution</th>
<th>Non-Polar Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔGpolar</td>
<td>ΔGnonpolar</td>
</tr>
<tr>
<td>β-amyloid</td>
<td>PMAQA</td>
</tr>
<tr>
<td>ΔGp</td>
<td>ΔGnp</td>
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</tr>
<tr>
<td>112.7</td>
<td>113.0</td>
</tr>
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</table>

The root mean square deviation (RMSD) of the backbone atoms calculated from 1 µs MD simulation of β-amyloid-PMAQA system showed an RMSD plateau with an average value =11 Å (Fig. 5a). A relatively small backbone RMSD (average value =8.8 Å) was observed for amylin-PMAQA system indicating a comparatively stable complex formation (Fig. 5c). The RMSD of PMAQA calculated from all-atoms depicted a nearly equal RMSD value =6.5 Å for both MD systems (Fig. 5d). Overall, the stable backbone with an average RMSD <2 Å over several hundreds of nanoseconds indicate a strong coupling between PMAQA and amylin or β-amyloid-1-40 over the ~µs time scale MD simulation. Further the root mean square fluctuation (RMSF) analysis of individual amino acids in β-amyloid-1-40 or amylin highlighted the potential PMAQA interaction regions. The amyloid aggregation core domains in both β-amyloid-1-40 (KLRF) and amylin (NFGAIL) depicted an RMSF value lower than their corresponding average values as indicated in Fig. 5d. In amylin, residues 7-16 showed the lowest RMSF values that folded to a stable α-helical conformation. This indicates the PMAQA interaction restrain the structural and dynamic properties of amylin. The tight coupling of PMAQA close to the proximity of NFGAIL region in amylin followed by restriction in protein structural rearrangement reveal mechanistic insights into PMAQA’s antagonist property in amylin aggregation. Further, the secondary structure analysis evolved from MD trajectories in amylin-PMAQA complex showed no significant secondary structural change during the MD simulation in amylin (Fig. 5S, bottom). On the other hand, a substantial secondary structure change was observed in β-amyloid-1-40 complexed with PMAQA including the induction of an antiparallel β-sheet along the terminal
residues (Figs. S5a and S5, top). The interaction of PMAQA with the centrally located residues of β-amyloid-1-40 such as Glu22, Asp23, Val24, Ser26 and Asn27 induced a random-coil and helix conformation in β-amyloid-1-40. Structural changes in β-amyloid-1-40 by affecting these central residues by mutation, disruption of the D23-K28 salt-bridge or binding of hexapeptide of genetic β-amyloid-1-40 variants has been reported previously to be crucial in accelerating β-amyloid-1-40 aggregation and induction of terminal β-structure.65–68 The charge-charge interaction between PMAQA (−NR3+) and Glu22/Asp23 in β-amyloid-1-40 could interfere with the early oligomer morphology and distribution of large β-amyloid-1-40 aggregates as observed in SEC (Fig. S3).69 The binding of PMAQA to Glu22 and Val24 also affects the hydrophobic interaction between Val24 and Lys28 and electrostatic interaction between Lys28 and Glu22/Asp23.70

We next monitored the aggregation kinetics of amylin on real-time using HS-AFM as the results presented above indicated an antagonistic behavior of PMAQA on amylin aggregation (Fig. 6). The fibrillation kinetics of freshly prepared amylin monomers was accelerated by adding preformed sonicated amylin amyloid seeds as described in the Experimental Section. As shown in Figure 6a (top trace), the addition of amylin monomers to pre-incubated amylin amyloid seeds (at t=0 s) exhibited a steady growth of fibers (Movie S1). Seeding reaction (or fibril growth) and de novo nucleation (binding of additional fibril seeds to the stage) were observed (Fig 6a). At 1000 s, we observed substantial growth in fiber morphology, de novo nucleated amylin fibers and several fibers were stopped growing after encountering with other fibers at their growing end (Fig 6a, Movie S1). The growth of the selected amylin fiber (Fig. 6a, rectangle) in the absence of PMAQA are represented as kymographs as a function of time in one direction. The kymograph showed a substantial growth on the free end of the selected amylin fiber (Fig. 6b). These observations agree with the reported observations for other amyloidogenic peptides including β-amyloid.69–71 Remarkably, in the presence of equimolar PMAQA, both amylin fibril growth and de novo nucleation were found to be blocked (Fig. 6c, Movie S2). In addition, no significant difference in the fiber length and population were observed between 0 s and 1350 s (Fig. 6c). Kymographs of amylin fibrillation in the presence of PMAQA showed no growth and indicates the polymer blocks the recruitment of amylin monomers to the fiber end to proceed the seeding reaction (Fig 6d). These distinct morphological features observed on both bare mica and APTES-mica suggest that the electrostatic interaction between the sample and the stage did not affect amylin’s aggregation and the interactions with PMAQA. The HS-AFM experimental results, therefore, revealed
the inhibitory activity of PMAQA on amylin aggregation at a molecular-level, which is consistent with other experimental results reported in this study.

We further characterized the morphology of β-amyloid-1-40 and amylin species present under various sample conditions (see the Experimental section). TEM image of 5 μM PMAQA showed very small particles (Fig. 7a), whereas freshly dissolved β-amyloid-1-40 monomers (5 μM) after continuous agitation at room temperature for 24 hours showed fibril morphology (Fig. 7b). On the other hand, β-amyloid-1-40 monomers (5 μM) incubated with an equimolar concentration of PMAQA exhibited amorphous-like aggregates (Fig. 7c). This correlate with the observed ThT fluorescence results that showed a substantial reduction in fluorescence intensity (Fig. 1a). We next examined the effect of PMAQA on β-amyloid-1-40 aggregation in the presence of amyloid seeds. TEM confirmed the formation of short fibrils when sononating matured fibers for 2 hours (Fig. 7d). We did not observe a significant change in the sonicated fiber morphology incubated with equimolar PMAQA for 24 hours in the absence of peptide monomers (Fig. 7e). However, a remarkable difference in β-amyloid-1-40 morphology was observed when a self-seeding reaction was performed in the absence or presence of PMAQA (Fig. 7f and g). The self-seeding reaction presented amyloid fiber growth in the absence of PMAQA (Fig. 7f), whereas in the presence of PMAQA short fibers and amorphous-like fiber morphology were identified (Fig. 7g). Unlike β-amyloid-1-40, amylin monomers (5 μM) incubated with equimolar PMAQA exhibited no fibers (Fig. 7i) which correlate to the ThT and CD results (Figs. 1b and 2b). Moreover, sonicated amylin fibers incubated with 5 μM PMAQA for 24 hours in the absence of monomers showed a relatively thick fibril morphology as compared to the short-sized sonicated fibers (Fig. 7j). Interestingly, sonicated amylin fibers in presence of equimolar peptide monomers and PMAQA showed no fibril growth which correlates the HS-AFM observations (Fig. 7k). A quantitative analysis of the widths of sonicated fibers (Fig. 7j) using ImageJ plugin presented an average fiber width of ~10.79 nm. In the presence of PMAQA, the sonicated fibers depicted an average width of ~15.69 nm indicating the possibility of PMAQA binding (Fig. 7l). Overall, the TEM analysis showed the formation of distinct morphological species of β-amyloid-1-40 and amylin in the presence or absence of PMAQA. These results further highlighted the opposite behaviors of PMAQA on the aggregation of β-amyloid-1-40 and amylin as observed in other biophysical experiments.

Conclusions

In conclusion, we have demonstrated the counter activities of a cationic PMAQA polymer on two different amyloidogenic peptides that are connected to AD and T2D. At subsiodynamic concentrations, PMAQA showed significant inhibitory activity in amyloid aggregation; whereas it significantly accelerated β-amyloid-1-40’s aggregation by quickly altering the equilibrium state of β-amyloid-1-40 from an unfolded structure to a β-sheet structure. Our mechanistic study provides insights into the binding of PMAQA to β-amyloid-1-40 or amylin at atomic-level that could be helpful in understanding the modulation of peptide self-assembly and could aid in potential inhibitor designing. We believe that the opposite aggregation kinetics of two different amyloidogenic proteins in the presence of a cationic polymer delineated in this study are likely to open avenues to test their potential therapeutic activities against an array of amyloid proteins involved in other human amyloid diseases by controlling functionalization of the polymer’s chemical property.
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