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Influence of dendrimer's structure on its activity against amyloid fibril formation

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Abstract

Inhibition of fibril assembly is a potential therapeutic strategy in neurodegenerative disorders such as prion and Alzheimer's diseases. Highly branched, globular polymers—dendrimers—are novel promising inhibitors of fibril formation. In this study, the effect of polyamidoamine (PAMAM) dendrimers (generations 3rd, 4th, and 5th) on amyloid aggregation of the prion peptide PrP 185–208 and the Alzheimer's peptide Aβ 1–28 was examined. Amyloid fibrils were produced in vitro and their formation was monitored using the dye thioflavin T (ThT). Fluorescence studies were complemented with electron microscopy. The results show that the higher the dendrimer generation, the larger the degree of inhibition of the amyloid aggregation process and the more effective are dendrimers in disrupting the already existing fibrils. A hypothesis on dendrimer–peptide interaction mechanism is presented based on the dendrimers' molecular structure.

Keywords: Dendrimer; Prion; Alzheimer; Amyloid peptide; Aggregation

Amyloid fibrils are aggregates of normally soluble peptides or proteins. There is a group of diseases that are characterized by the deposition of amyloid fibrils. Among them there are neurological disorders such as Alzheimer's and prion diseases. Thus, inhibition of fibril assembly is a potential strategy for therapeutic intervention. It has been recently shown that polyamidoamine and polypropyleneimine dendrimers are promising candidates for the treatment of prion diseases [1,2]. These relatively novel macromolecules are globular and are characterized by a densely packed surface. Due to their specific structure they are suitable for a variety of biomedical applications. Dendrimers promote the clearance of pre-existing PrP^{Sc} (the abnormally folded prion protein, which forms amyloid fibrils). Dendrimers also prevent the conversion of the normal cellular PrP^{C} into PrP^{Sc} [1,2]. These branched polyamines are the first class of compounds that have been shown to be able to cure a prion infection in living cells. This fact received considerable interest and other types of dendrimers were tested as possible anti-prion agents [3,4].

Amyloid fibrils can be produced in vitro by exposing disease-associated peptides to destabilizing conditions. We have chosen this approach with the aim of contributing to the molecular characterization of the interactions between dendrimers and peptides.

We have used the third, fourth, and fifth generation of polyamidoamine dendrimers (PAMAM G3, PAMAM G4, and PAMAM G5) in order to study how dendrimers' structure and size determine their effect on amyloid formation. Dendrimers are built in a cyclic manner from a central core molecule that is surrounded by layers of branched monomers. The more layers are attached, the higher the so-called generation. As generation increases, the amount of surface groups increases too, so the shape of the dendrimer changes from flat and ellipsoidal to globular [5]. In case of polyamidoamine dendrimers ethylenediamine is the core molecule and branched units are constructed from

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Table 1
Characterization of used dendrimers [32]

Name, generation	Terminal groups	Number of terminal groups	Molecular weight [Da]	Diameter [nm]
PAMAM, G3	$-NH_2$	32	6,909	3.6
PAMAM, G4	$-NH_2$	64	14,215	4.5
PAMAM, G5	$-NH_2$	128	28,826	5.4

both methyl acrylate and ethylenediamine [6]. A summary of the characteristics of the dendrimers used in the present study is given in Table 1.

As a continuation of our previous studies we have chosen Alzheimer's peptide Aβ 1–28 and a segment of prion protein PrP 185–208 [7]. A structural homology has been recently described for these two sequences and a recent computational study has shown that residues 180–193 are one of the fibrilization sites in PrP [8,9].

Dendrimers were studied in a system containing heparin as a model glucosaminoglycan. It has been found that amyloid fibrils in vivo are normally associated with GAGs, and both PrP 185–208 and Aβ 1–28 have been previously shown to aggregate in the presence of heparin [10,11].

Materials and methods

Materials. Synthetic peptides Aβ 1–28 [DAEFRHDSGYEVHHQ KLVFFAEDVGSNK] and PrP 185–208 [KQHTVTTTTKGENFTET DVKMMER] were purchased from JPT Peptide Technologies GmbH (Germany). Stock peptide solutions were kept in aqueous buffer at pH 7.5. Thioflavin T (T-3516) and heparin–sodium salt (H-4784) were purchased from Sigma Chemical Company. Dendrimers PAMAM G3, PAMAM G4, and PAMAM G5 were obtained from Dendritic NanoTechnologies Inc. (USA) and dissolved in aqueous buffer. All other chemicals were of analytical grade. Water used to prepare solutions was double-distilled.

Formation of amyloid fibrils—ThT assay. The process of aggregation was monitored using the dye thioflavin T (ThT), whose fluorescence depends on the presence of amyloid structures [12,13]. A stock solution of peptide (1.2 mmol/l) in Tris buffer, pH 7.5, was diluted to a final concentration of 50 μmol/l. Then ThT and heparin were added (final concentrations of 35 μmol/l and 0.041 mg/ml, respectively) and pH was adjusted to 5.5 with aliquots of HCl. Fluorescence measurements were performed at 37 °C upon continuous shaking using a microplate reader (Wallac 1440 VICTOR^3 V Multilabel Counter from Perkin-Elmer). Aggregation kinetics were monitored by measuring the fluorescence intensity every 375 s using 450-nm excitation and 490-nm emission filters.

Electron microscopy. Ten microliters of sample from the fluorescence experiment (see previous paragraph) was placed on a carbon 400 mesh grid. It was dried and the excess of solution was removed with a filter paper. The sample was stained with 2% uranyl acetate for 2 min, dried, and then viewed using a Hitachi H-7000 electron microscope.

Results

The fluorescence of thioflavin T is normally used to monitor the formation of amyloid fibrils. Figs. 1 and 2 show the fluorescence variation of ThT for Aβ 1–28 and PrP 185–208 in the absence and presence of increasing concentrations of dendrimers. The time-dependent increase in ThT fluorescence follows a sigmoidal curve typical of a nucleated polymerization reaction. Peptide monomers slowly combine to form non-fibrilar structures known as nuclei (lag phase). Addition of peptide monomers to these nuclei and combination of nuclei, together with a conformational transition which implies the formation of fibrilar β-sheet structures, results in the so-called elongation phase

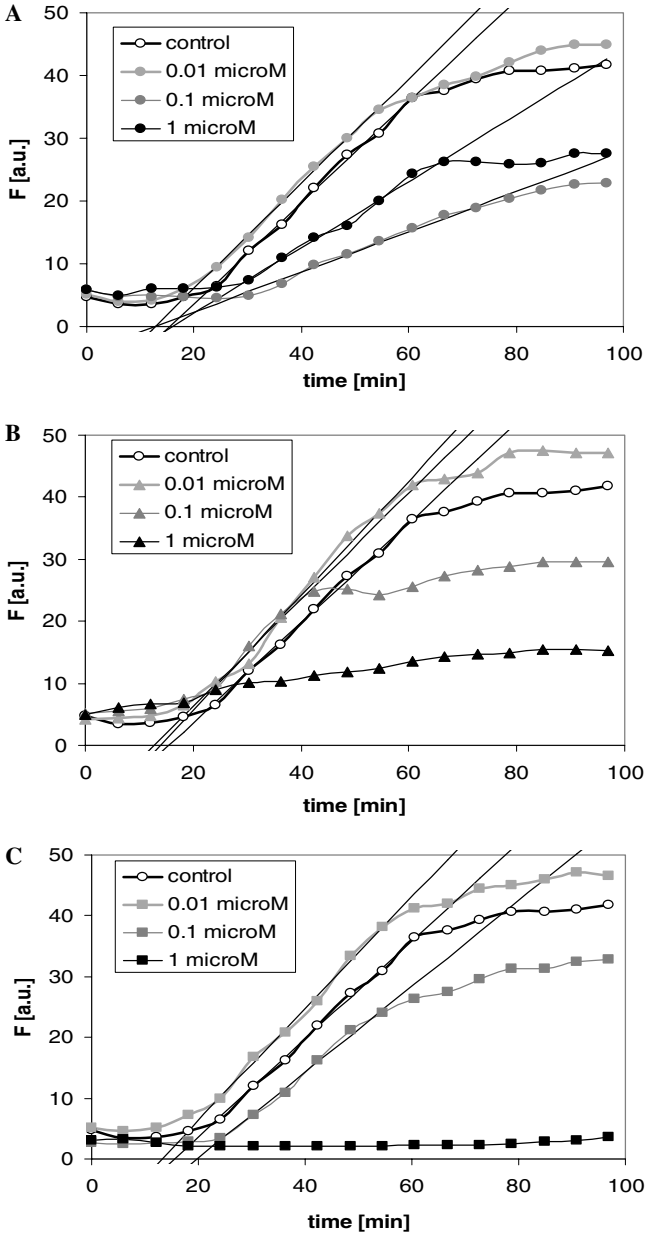

Fig. 1. Changes in fluorescence of ThT during the aggregation processes of Aβ 1–28 peptide in the presence of PAMAM G3 (A), PAMAM G4 (B), and PAMAM G5 (C).

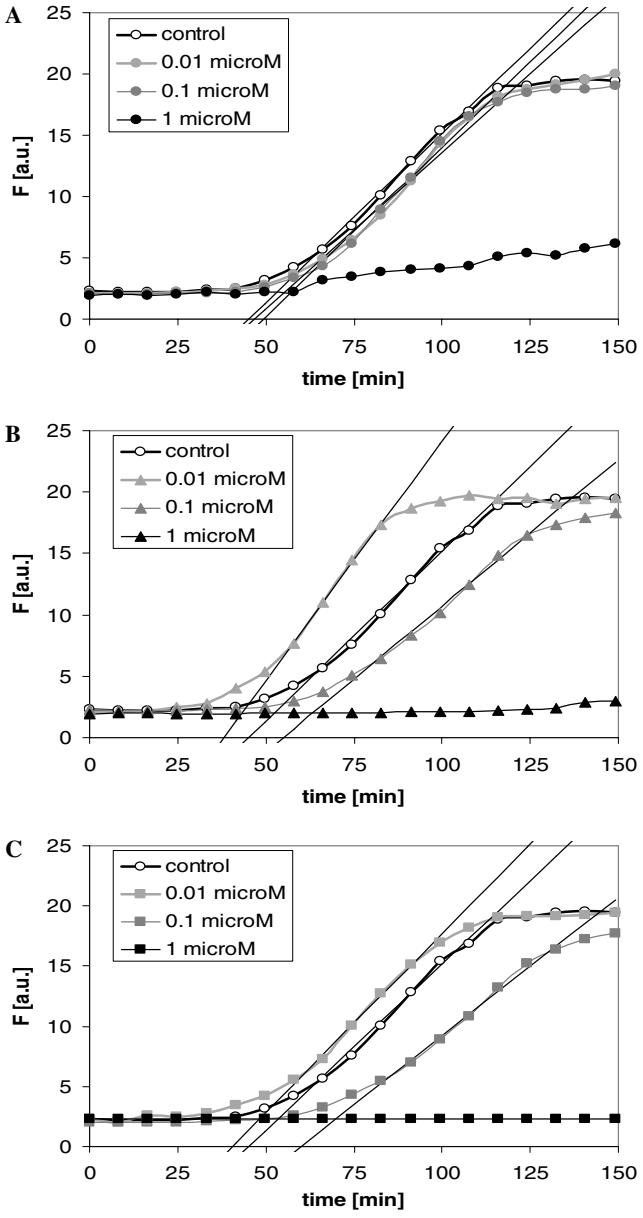

Fig. 2. Changes in fluorescence of ThT during the aggregation processes of PrP 185–208 peptide in the presence of PAMAM G3 (A), PAMAM G4 (B), and PAMAM G5 (C).

(the faster, exponential part of the sigmoid). In this latter process, breakage of long fibrils giving shorter structures that can act as seeds may be an important part of the mechanism [14]. From the graphs, the duration of the nucleation phase can be estimated by determining the point where the prolongation of the exponential part of the sigmoid cuts the time axis [15]. Fig. 1 shows that in the case of Aβ 1–28 the duration of the nucleation reaction (lag phase) does not change much, compared to the control, in the presence of PAMAM dendrimers. At low dendrimer concentration (0.01 μmol/l) there is no effect on the elongation rate (measured as the slope of the exponential part of the sigmoid) or at most a slight increase (in agreement with previous reports [7]). PAMAM G3 dendrimers, however, clearly slow down the elongation reaction when present at 0.1 and 1 μmol/l, significantly reducing as well, the amount of final fibrils formed. The effect of PAMAM G4, and PAMAM G5 at 0.1 μmol/l on the elongation rate is much less evident than for PAMAM G3, although at 1 μmol PAMAM G4 and PAMAM G5 completely inhibit the formation of fibers in the monitored time interval. Low generation PAMAM dendrimers have therefore a clear

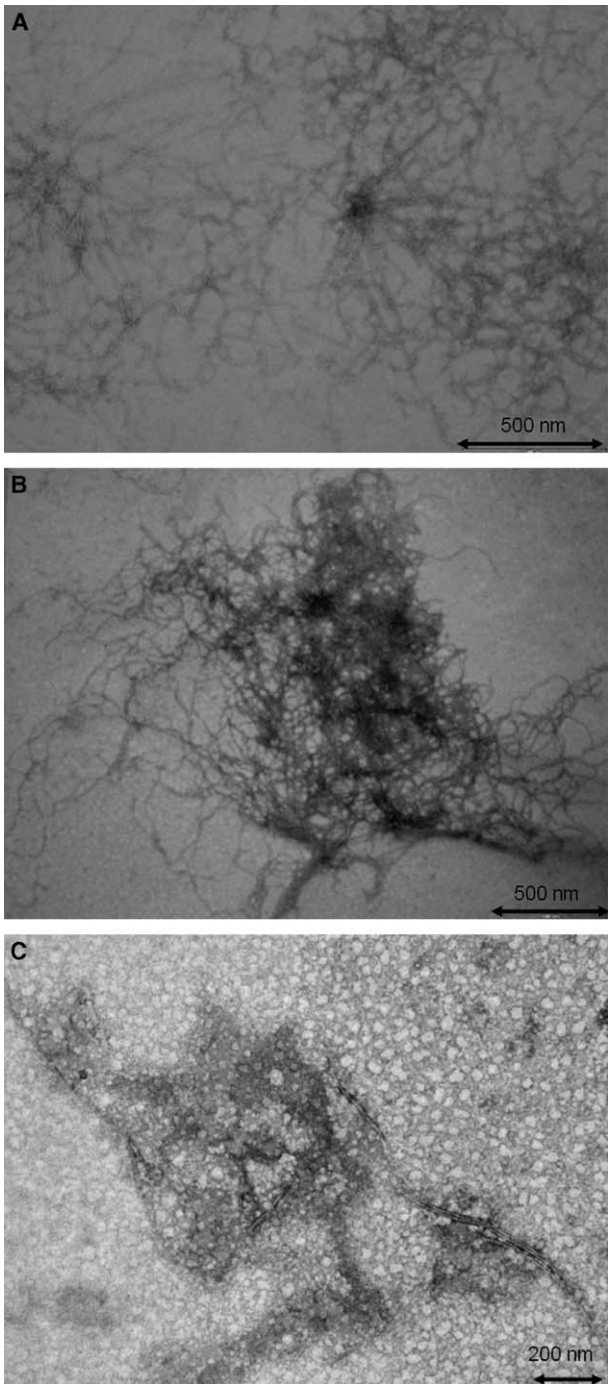

Fig. 3. Electron micrographs of PrP structures in the absence of dendrimers (A), in the presence of 1 μmol/l PAMAM G3 (B) and 1 μmol/l PAMAM G5 (C).

influence on the elongation rate of Aβ 1–28 aggregation, an effect which is less pronounced for higher generations (4th and 5th). In general (for all PAMAM G3, PAMAM G4, and PAMAM G5) there is a clear effect on the final concentration of fibrils: the higher the dendrimer concentration and the higher the generation, the smaller the amount of fibrils formed.

For PrP 185–208, the duration of the nucleation phase is in general slightly more than double that of Aβ 1–28 (Fig. 2). PAMAM G3 at 0.01 and 0.1 μmol/l has no effect on the aggregation rate or in the final amount of fibrils. 0.01 μmol/l of PAMAM G4 or PAMAM G5 seems to accelerate the whole process whereas 0.1 μmol/l does slow it down. In both cases the final amount of fibrils does not change. In all cases however (PAMAM G3, PAMAM G4, and PAMAM G5) 1 μmol/l dendrimer completely inhibits fibril formation in the monitored time interval.

In order to complement the ThT results, we analyzed the final products by electron microscopy (Fig. 3). The characteristic long, interweaved fibrils were observed for the control sample. In the presence of PAMAM G3 dendrimers, fibrils were not as straight and they were organized in clumps. Among the fibrils amorphous structures were noticeable. A clearly smaller amount of fibrils was visible in the presence of PAMAM G5, where amorphous structures predominated.

Finally, in order to check the dendrimers' ability to disaggregate amyloid fibrils we added dendrimers at increasing concentrations into samples where aggregates had already been formed and we monitored the changes in ThT fluorescence (Fig. 4). It turned out that all dendrimers were able to disrupt aggregates. The higher the generation, the lower the concentration needed to achieve the disaggregating effect. The concentration that corresponds to 50% decrease in the content of amyloid fibrils (c_{50}) was 0.2, 0.5, and 1.1 μmol/l for PAMAM G5, PAMAM G4, and PAMAM G3, respectively.

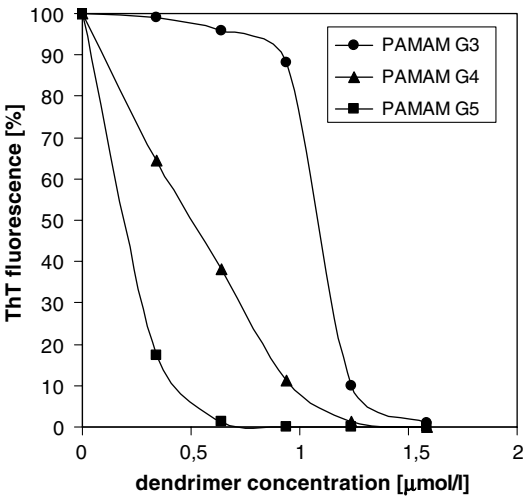

Fig. 4. Changes in fluorescence of ThT during the disaggregation process for PrP 185–208 peptide upon dendrimers.

Discussion

The presence of abnormal aggregates, so-called amyloid fibrils, is observed in several neurodegenerative disorders such as prion diseases and Alzheimer's disease. The search for an effective treatment for these disorders is an up-to-date issue. Alzheimer's dementia is a disease that threatens to overwhelm a health care system in the developed world. In contrast to Alzheimer's disease, prion diseases can be transmitted from one organism to another. It was believed that the process cannot occur between species. Unfortunately a new variant of Creutzfeldt–Jakob disease (nvCJD) showed that this barrier was crossed. Several lines of evidence prove that a link between the nvCJD outbreak and a preceding epidemic of bovine spongiform encephalopathy (BSE) exists [16]. Although it is too early to predict the number of nvCJD cases that might eventually arise, it is clear that effective therapeutics for prion diseases are urgently needed. It seems that inhibition of fibril assembly can be a potential strategy for therapeutic intervention.

Several inhibitors (e.g., porphyrines, phthalocyanines, and polycyclic aromatics) have been recently identified [17–19]. Among these two main types can be distinguished: kinetic and thermodynamic inhibitors [14]. They can be identified by analyzing the shape of the kinetics curve in comparison to a control curve. In the case of kinetic inhibitors the final amount of fibrils is unchanged, the lag time however varies, but the control and inhibitor curves cannot be superimposed. We observed this type of kinetic inhibition for PrP 185–208 in the presence of 0.1 μmol/l PAMAM G4 and 0.1 μmol/l PAMAM G5 (1 μmol/l completely inhibited amyloid formation in the monitored time interval). Thermodynamic inhibitors on the other hand do not affect amyloid formation rate but the final amount of fibrils is reduced. We observed this effect in the case of Aβ 1–28 aggregation in the presence of PAMAM G4 and PAMAM G5, which had only a small effect on both the nucleation and elongation rates but clearly decreased the sigmoid plateaus. PAMAM G3 did not affect the nucleation rate (lag phase duration) but significantly lowered the elongation rate (straight line slopes in Fig. 2A). PAMAM G3 reduced as well the final amount of fibrils, although to a lesser extent, compared with PAMAM G4 and PAMAM G5. Contrary to some specific inhibitors as, e.g., aplidine that blocks fibril formation for PrP 106–126 better than for Aβ 25–35 [20], dendrimers at higher concentrations (1 μmol/l) behaved similarly in the presence of both prion and Alzheimer's peptides.

Considering the different mechanisms that have been proposed for amyloid fibril formation, three different ways of inhibiting amyloidogenesis can be envisaged: by lowering the effective peptide concentration, by blocking the ends of growing fibrils, and by increasing the fibril breakage rate [14]. In order to interpret the effect of dendrimers, all these strategies should be considered (Fig. 5).

Dendrimers could lower the concentration of peptides that are capable of growing into fibrils by binding to the

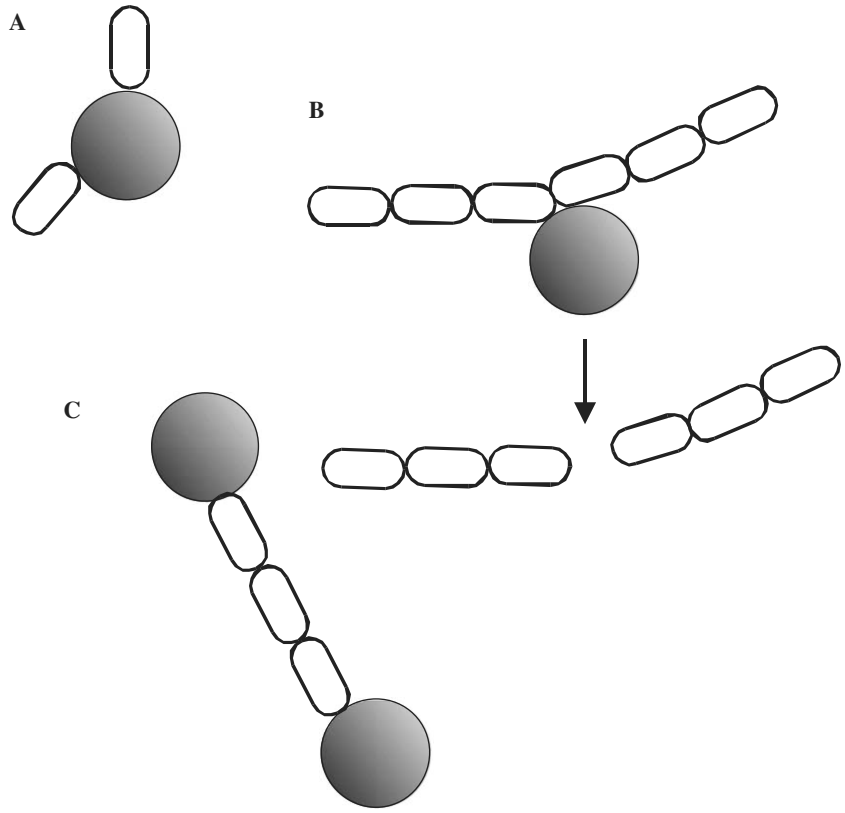

Fig. 5. Possible strategies of inhibition of amyloidogenesis by dendrimers: by binding to peptides (A), breaking fibrils (B), and blocking free ends of a fibril (C).

peptide monomers. It has been previously shown that dendrimers have an affinity for proteins, e.g., they create a layer on the surface of albumin and change its conformation [21]. Dendrimers can also affect proteins' functions, e.g., change an enzyme activity [22]. However, this inhibition mechanism is less likely to be observed under our experimental conditions since it is believed that this group of inhibitors is not effective if fibrils grow exponentially according to a second kinetic stage [14].

It can be observed in Figs. 1 and 2 that at low dendrimer concentration (0.01 μmol/l) fibril formation is accelerated. This behavior is typical of inhibitors that increase the fibril breakage rate. A moderated fibril breakage level in amyloidogenic processes may be responsible for speeding up the formation of aggregates by creating new free ends that can expand upon an attachment of new peptides. However, if breakage is very fast it can progressively break down all fibrils until only monomers are left. Therefore, if fibril breakage is the predominant mechanism of inhibition, then inhibitors administered in low doses can in fact accelerate fibrilogenesis by providing a larger amount of ends that serve as sites of replication. In higher doses however these inhibitors can be effective. Such a mechanism was previously found for Congo red [23]. Dendrimers' ability to disrupt already existing fibrils is illustrated in Fig. 4. The smaller the dendrimers, the higher the concentration needed to disrupt the aggregates. This is in a good agreement with the reported effect of poly-L-lysine on amyloid aggregates [24]. Poly-L-lysine is a potent dissolver but its monomer L-lysine is not effective due to its small size.

Finally, amyloid formation can be inhibited by blocking the fibril ends. Usually effective end-blocking inhibitors are very similar to amyloid proteins [25]. This guarantees that they can bind to the fibril end, and the subtle differences in their structure prevent further fibril elongation. Although dendrimers have been called 'artificial proteins' and they can mimic some protein functions [26], it is obvious that they do not bear much resemblance to proteins. Nevertheless, it is possible that they bind unspecifically to fibrils affecting their growth. This hypothesis is supported by electron microscopy observations (Fig. 3). In the presence of low generations of dendrimers (PAMAM G3) fibrils seem to be more curved and they seem to tend to be organized in clumps. Fibril organization may have been somehow changed by the presence of dendrimers. Dendrimers possess many end groups on the surface. Each of them can interact with peptides or fibrils. The star structure of a dendrimer could be responsible for the formation of clumps (Fig. 6). Higher generations (PAMAM G5) seem to have been more effective in their interactions with monomeric peptides and/or breaking fibrils, since less fibrils were detected by electron microscopy (Fig. 3C). A schematic description of the way in which big dendrimers could hamper the formation of long fibrils is presented in Fig. 7. Besides a reduced amount of fibrils, peptides formed amorphous aggregates in the presence of PAMAM G5.

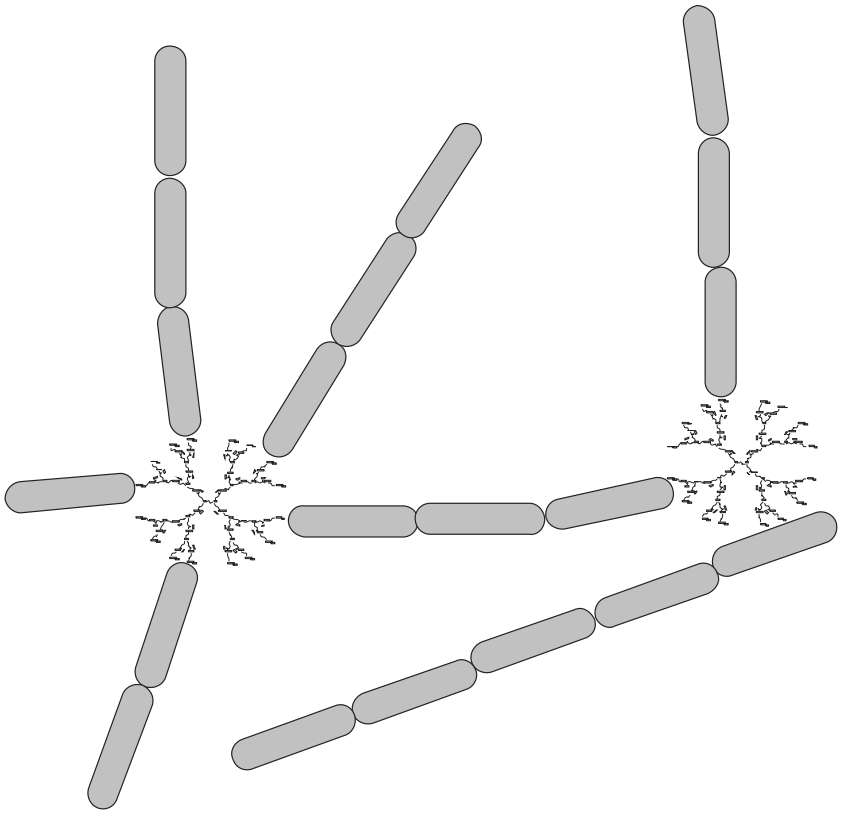

Fig. 6. The model of interaction between peptides and low generations of PAMAM dendrimers.

Amorphous random coil aggregates from Alzheimer peptides have been described at acidic pH of 5–6 [27,28]. ThT does not fluoresce in the presence of amorphous aggregates [13,29].

In our studies, heparin was added to all samples. Heparin is a mixture of unbranched anionic mucopolysaccharides joined by glycosidic linkages. It was previously shown that the presence of heparin in the medium at pH 5.5 shortened the lag time and increased the slope of the sigmoid [7]. It has been established that GAGs promote fibril formation and stabilize existing fibrils [30]. Since heparin is negatively charged and dendrimers possess cationic groups on the surface, it is obvious that electrostatic interactions between heparin and dendrimers can occur. However, it is important to notice that the role of dendrimers cannot be limited to the clearance of heparin from the system. If it were so, then we would not observe the acceleration the fibril formation process in the presence of low concentrations of dendrimers. Besides, it has been shown that Aβ 1–28 forms fibrils in the absence of heparin, although the process is considerably slower, and PAMAM G3 at high concentration was able to completely inhibit this process [7].

Generally, the bigger the dendrimer molecule, the higher the activity against fibrils was observed. It has been previously shown that polyamidoamine and polypropyleneimine dendrimers become more potent at eliminating PrP^{Sc} as they are more branched [1]. Thus, the inhibiting processes are correlated with the number of amino groups on the surface. However, it has been found that there is an optimum balance between the size of dendrimers and the number of end groups on their surface. The efficiency of phosphorus-containing dendrimers (which possess protonated terminal tertiary amines) against PrP^{Sc} increased according to the order: generation 3rd < generation 5th < generation 4th [31]. In our studies, 1 μmol/l PAMAM G5 was enough to completely inhibit the formation of fibrils. This concentration corresponds to a ratio of peptide:dendrimer of 50:1, and to a ratio of peptide:dendrimer's surface groups of 2.56. This illustrates how relatively low molar concentrations of dendrimers can be efficient due to the large number of surface groups. It is believed that the presence of amino groups is crucial, since it has been proven that hydroxy-terminated dendrimers are ineffective [1]. This means that electrostatic interactions between dendrimers and peptides are very important.

To summarize, it seems that dendrimers can interfere with amyloid formation by combining at least two inhibiting strategies: blocking fibril growth and breaking existing fibrils. However, their efficiency is limited and their action can result in the formation of amorphous, non-fibrillar aggregates. It is worth reminding that amyloid fibrils are only one type of aggregates that is very specific due to its highly organized 'misfolded' structure. This structure is responsible for the protease K resistance. Previous studies have shown that in the presence of dendrimers the resistant forms become sensitive to protease K [2]. The fact that dendrimers can render amorphous aggregates in amyloid

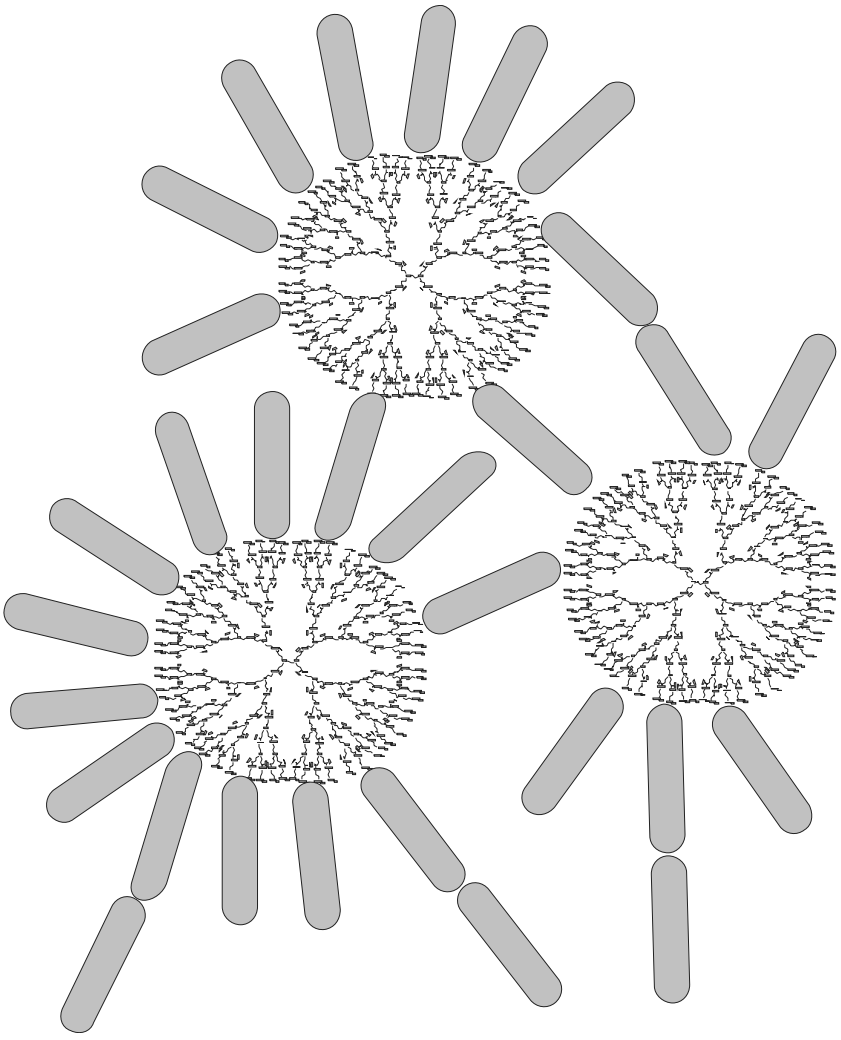

Fig. 7. The model of interaction between peptides and high generations of PAMAM dendrimers.

peptide suspensions is therefore worth considering in the path which leads to the design of successful pharmacological strategies.

Acknowledgments

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