Molecular Interactions of Dendrimers with Amyloid Peptides: pH Dependence

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Received March 11, 2006; Revised Manuscript Received May 1, 2006

The formation of amyloid plaques is a key pathological event in neurodegenerative disorders, such as prion and Alzheimer's diseases. Dendrimers are considered promising therapeutic agents in these disorders. In the present work, we have studied the effect of polypropyleneimine dendrimers on the formation of amyloid fibrils as a function of pH in order to gain further insight in the aggregation mechanism and its inhibition. Amyloid fibrils from prion peptide PrP 185-208 and Alzheimer's peptide Aβ 1-28 were produced in vitro, and their formation was monitored using the dye thioflavin T (ThT). The results showed that the level of protonation of His, Glu, and Asp residues is important for the final effect, especially at low dendrimer concentration when their inhibiting capacity depends on the pH. At the highest concentrations, dendrimers were very effective against fibril formations for both prion and Alzheimer's peptides.

Introduction

Dendrimers are a relatively new class of polymeric materials. Unlike linear polymers, they have a well-defined structure. They consist of a central core and branched monomers. The cyclic manner in which they are built results in a globular shape and a large number of terminal groups on the surface. The more layers of branched units are added, the higher the dendrimer generation. PPI dendrimers, used in this work, belong to the class of branched polyamines. Butylenediamine is used as a core molecule. These dendrimers are synthesized by a repetitive reaction sequence that involves Michael addition of acrylonitrile to a primary amino groups followed by hydrogenation of nitrile groups to primary amino groups.[1] The structure of the dendrimer is shown in Figure 1. Dendrimers have attracted much interest since their discovery due to the specific structure. They are promising materials as drug carriers and transfection agents.[2−7]

It has recently been shown that dendrimers have their own biological activity against scrapie prion proteins (PrP^{Sc}).[8,9] Prion diseases are fatal neurodegenerative disorders resulting in conformational changes from a normal cellular form of prion proteins (PrP^{C}) to an infectious scrapie isoform. The infectious form is rich in β-structure as a result of a transformation from unordered plus helical structures present on the nonpathological form of the protein. The β-form leads to the formation of fibrils—amyloid-like structures.

Several compounds have been reported as promising therapeutic agents in prion diseases.[10,11] Unfortunately, all of them have demonstrated only weak potential to stop prion propagation, and none of them have shown the ability to remove the pre-existing prions from an infected organism. Non-cytotoxic concentrations of amino-terminated dendrimers are able to purge PrP^{Sc} from scrapie-infected neuroblastoma cells in culture.[8] This is the first class of compounds that promotes the clearance of pre-existing PrP^{Sc}. Because there is a similarity between prion diseases and Alzheimer's disease (Alzheimer's disease is also associated with the formation of amyloid aggregates), the possibility exists that dendrimers can be therapeutic agents against this neurodegenerative disorder as well.

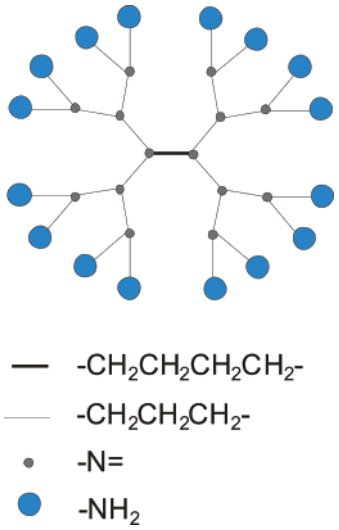

Figure 1. The structure of PPI dendrimer.

The aim of the present study was to monitor the effect of pH on the amyloid inhibiting activity of polypropyleneimine (PPI) dendrimers in order to further characterize the aggregation processes at the molecular level.

Understanding the driving forces behind dendrimer−peptide interactions, as well as structural motifs necessary for these interactions, can help to develop more efficient inhibitors against an amyloid formation and find the best conditions for their action. As a model for these studies, we have chosen Alzheimer's peptide Aβ 1-28 and a segment of prion protein PrP 185-208.[9] A structural homology has recently been described for this two sequences, which could play an important role in amyloidogenic process.[12]

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10.1021/bm060229s CCC: $33.50
Published on Web 06/03/2006

Materials and Methods

Materials. Synthetic peptides Aβ 1-28 [DAEFRHDSGYEVHHQKLVFFAEDVGSNK] and PrP 185-208 [KQHTVTTTTKGENFTETDVKMMER] 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. Polypropylenimine hexadecaamine dendrimer (DAB-Am-16), the third generation (-[CH_2-$CH_2N[(CH_2)_3N[(CH_2)_3N[(CH_2)_3NH_2]_2]_2]_2]_2$), was obtained from Sigma-Aldrich and dissolved in aqueous buffer. All other chemicals were of analytical grade. Water used to prepare solutions was double-distilled.

Formation of Amyloid Fibrils−Thioflavin T Assay. The process of aggregation was monitored using the dye thioflavin T (ThT), for which the fluorescence depends on the presence of amyloid structures.[13,14] 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 4.9, 5.4, or 5.9 with aliquots of HCl. Fluorescent measurements were carried out with a Perkin-Elmer LS-50B spectrofluorimeter. Experiments were performed at 37 °C upon continuous stirring. Kinetics of aggregation was monitored by recording a fluorescence intensity. The excitation and emission wavelengths were 450 and 490 nm, respectively. The excitation and emission slit widths were set to 5 nm.

Results

Formation of Amyloid Fibrils−Thioflavin T Assay. Since ThT fluorescence is sensitive to the presence of amyloid fibrils, it can serve as a good indicator of fibril formation. In our experiments, the formation of amyloid fibrils occurred in vitro, and the process was monitored over time. Sigmoidal curves typical of a nucleated polymerization reaction were observed. Analyzing the shape of the curve enables to compare two important parameters: the reaction rates (the nucleation rate given by the extension of the lag phase and the elongation rate given by the exponential part of the sigmoid) and the final concentration of amyloid fibrils (the level of fluorescence reached at the plateaus). Another advantage of the ThT assay is the possibility to perform experiments in different conditions, e.g., by changing pH.

Fibril formation has previously been shown to be modulated by several factors including pH.[15] We checked the impact of pH in the range from 4.9 to 5.9 on the aggregation process of the prion peptide PrP 185-208 and the Alzheimer peptide Aβ 1-28 (Figure 2). At pH higher than 6.0, no aggregation was observed in the monitored time interval. When pH was lower than 4.9, the process of aggregation was so fast that it was impossible to follow its kinetics. For PrP 185-208, the higher the pH is, the shorter the lag time, and the lower the final fibril yield. In case of Aβ 1-28, the pH dependence was not so consistent. The differences in the shape of the curves obtained at pH 5.4 and 5.9 were not big, but the lowest pH (4.9) caused a drastic acceleration of fibril formation. Moreover, in the case of Aβ 1-28, the lower the pH, the more fibrils are formed. For the same pH conditions as those described in Figure 2, we checked the influence of PPI dendrimers at four different concentrations (Figures 3 and 4). The highest concentration (4 μmol/L) caused a total inhibition in all experiments. The most interesting observation was that PPI at 1 μmol/L can either enhance or hamper fibril formation depending on the pH. At this concentration, we also observed an opposite effect for PrP 185-208 and Aβ 1-28. PPI decreased the final amount of Aβ 1-28 aggregates at the highest pH and PrP 185-208 aggregation

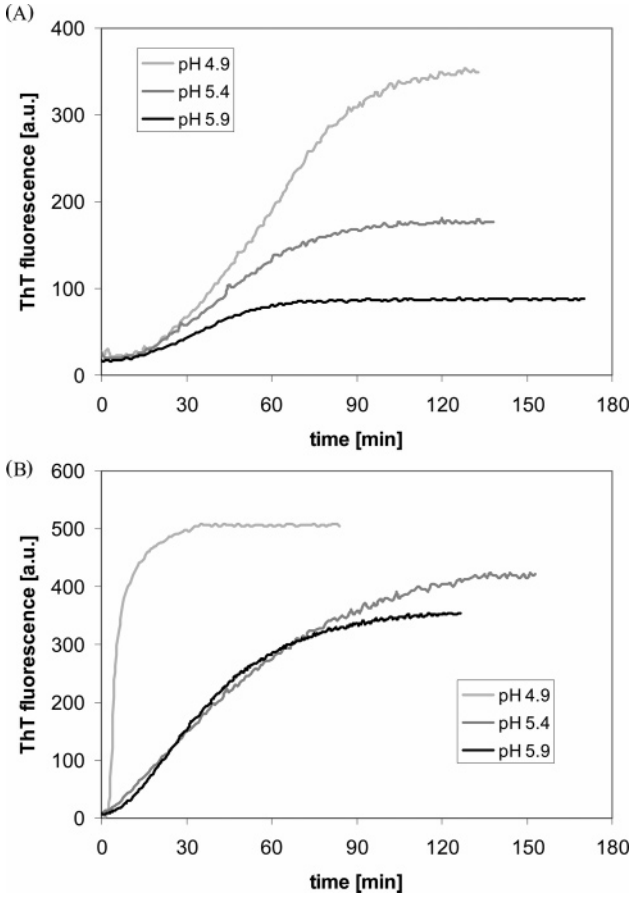

Figure 2. Changes in fluorescence of ThT during the aggregation processes of PrP 185-208 (A) and Aβ 1-28 (B).

at the lowest pH. The inhibiting property was observed for the lowest pH (4.9) in the case of PrP 185-208 and for the highest pH (5.9) in the case of Aβ 1-28. Moreover, sometimes the rate of amyloidogenesis was lower in the presence of 1.5 μmol/L of dendrimers than in the presence of dendrimers at concentration 2 μmol/L.

To check dendrimers' ability to disaggregate amyloid fibrils, we added dendrimers at increasing concentrations to samples where aggregates of PrP 185-208 had already been formed (Figure 5). First, we observed a slow but steady decrease of ThT fluorescence followed by a dramatic fall in the concentration range 1.25−1.75 μmol/L. This range corresponds to concentrations that significantly slowed de novo formation.

Effect of pH on the Charge Carried by Dendrimers and Peptides. There are some discrepancies in the value of pK for polyamidoamine dendrimers' amino groups, ranging from 6.85 to 9.00.[16,17] To our knowledge, this parameter has not been estimated for PPI dendrimers, but it is reasonable to assume that it will not differ significantly. The extent of ionization of the amino groups in PPI dendrimers was estimated using the Henderson−Hasselbalch equation

$$\mathrm{pH} = \mathrm{p}K + \log \frac{[\mathrm{NH_2}]}{[\mathrm{NH_3^+}]} \qquad (1)$$

In the studied pH range, when pK equaled 9.0, all amino groups were charged, and almost all (90−99%) for pK 6.85.

Electrostatic calculations for peptides were done using D. Bashford's MEAD program.[18] In the protein data bank, the PDB IDs for the peptides were found.[19] It was 1AMB for Aβ1-28.[20] Since PrP sequence 185-208 does not exist in the data bank,

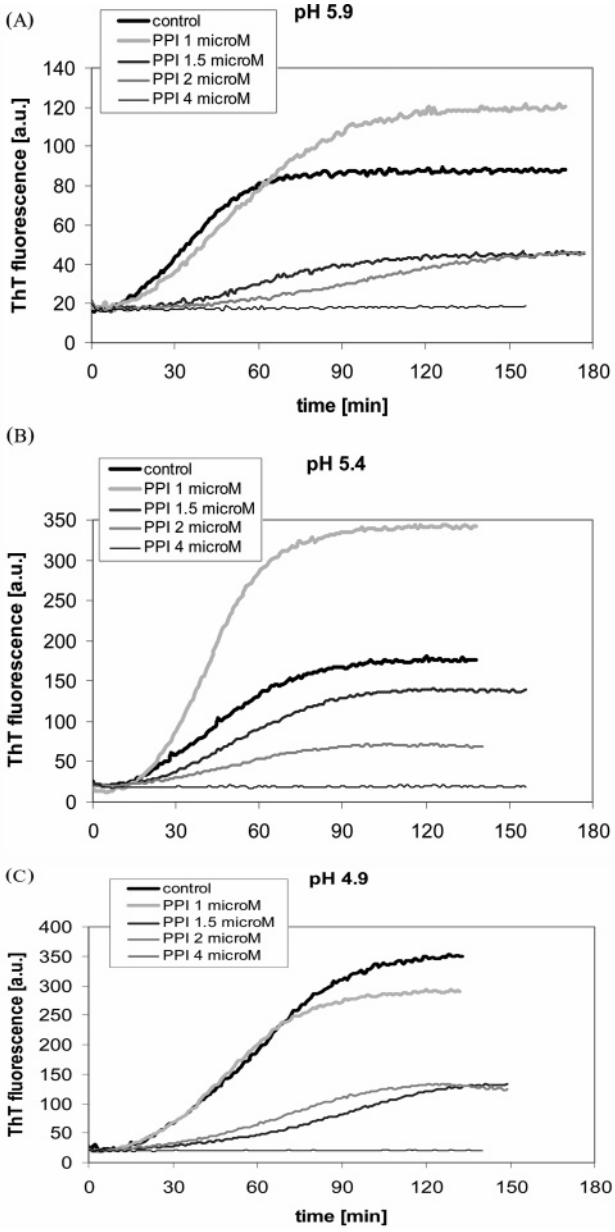

Figure 3. Changes in fluorescence of ThT during the aggregation processes of PrP 185-208 in the presence of dendrimers at pH 5.9 (A); pH 5.4 (B); pH 4.9 (C).

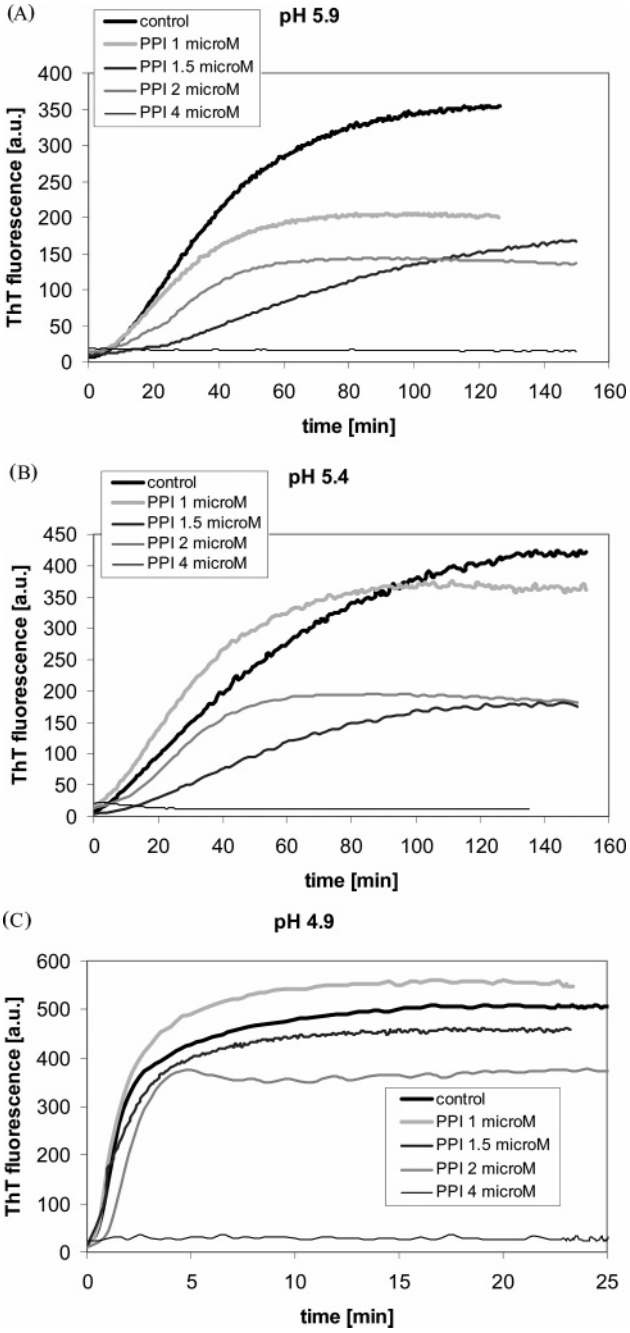

Figure 4. Changes in fluorescence of ThT during the aggregation processes of Aβ 1-28 in the presence of dendrimers at pH 5.9 (A); pH 5.4 (B); pH 4.9 (C).

calculations were made for a bigger fragment PrP 121-231 (PDB ID 1AG2).[21] Both the intrinsic pK of the individual residues in the peptide (pK_{int}) and the estimated apparent pK for the same residues (p$K_{1/2}$) were calculated, and results are summarized in Table 1. The levels of protonation of acidic and alkaline groups in amino acid residues were calculated according to the Henderson−Hasselbalch equation. Figure 6 shows the results for the residues that significantly change their protonation state in the studied range of pH. The effect of pH on the charge carried by peptides was illustrated in Figures 7 and 8.

Discussion

The aim of this work was to study the capacity of dendrimers to interfere with amyloidogenic processes involving prion and Alzheimer's peptides and how this capacity depends on pH.

Amyloid formation is a key pathological feature in a group of neurodegenerative disorders such as Alzheimer disease and prion diseases. Amyloid fibrils are polymeric forms of proteins. They are rich in β-sheet structures. Amyloid fibrils can be produced in vitro by subjecting prion or Alzheimer peptides to special conditions. Amyloidogenicity of prion peptides depends on a balance between solubility and insolubility, e.g., a charge at position 111 in PrP 106-126 was found to be important to retain adequate solubility to ensure fibrillation.[22] It is known that amyloid formation is favored by a charged C-terminus in the prion peptide 106-126,[23] whereas amidation of C-terminal Gly-126 resulted in a significant decrease of fibril formation.[22] A similar fibril-promoting effect of a free C-terminal carboxylic acid was found for Alzheimer's peptide.[24]

The ability of these peptides to form β-amyloid aggregates can also be modulated by pH. β-Sheet content in prion peptides is enhanced at low pH compared to neutral pH.[25] This is in good agreement with the fact that prion replication may occur

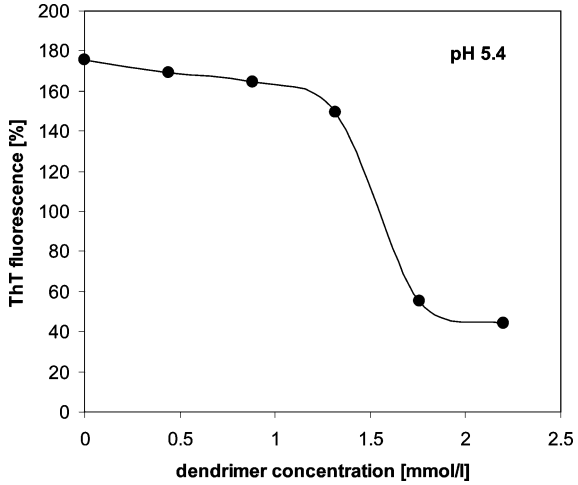

Figure 5. Changes in fluorescence of ThT during the disaggregation process for PrP 185-208 peptide upon dendrimers at pH 5.4.

Table 1. Intrinsic p*K*s of the Residues (pK_{int}) and Estimated Apparent pKs of the Residues for Aβ 1-28 and PrP 185-208

Aβ 1-28			PrP 185-208		
	pK_{int}	p$K_{1/2}$		pK_{int}	p$K_{1/2}$
NT-ASP-1	3.264	6.134	LYS-185	10.175	10.543
ASP-1	4.634	1.096	HIS-187	6.280	5.682
GLU-3	4.134	3.562	LYS-194	10.150	10.590
ARG-5	12.133	12.987	GLU-196	5.905	4.590
HIS-6	6.607	7.154	GLU-200	4.210	3.856
ASP-7	4.763	4.664	ASP-202	4.244	1.898
GLU-11	4.186	3.715	LYS-204	9.640	11.147
HIS-13	7.026	6.358	GLU-207	5.486	5.036
HIS-14	3.793	3.868	ARG-208	10.957	12.499
LYS-16	9.636	9.734			
GLU-22	4.801	4.937			
ASP-23	4.157	4.055			
LYS-28	10.499	10.883			
CT-LYS-28	4.386	4.117			

in cellular compartments that are characterized by an acidic environment, such as lysosomes and endosomes. Our results have shown that the lower pH, the faster the amyloidogenesis, but the pH dependence has been found to be different for PrP 185-208 and Aβ 1-28. In the case of Aβ 1-28 at pH 4.9, the process was extremely fast. This is consistent with the fact that for this peptide lowering the pH from neutral to 5.5 is sufficient to trigger fibril formation.[26] This feature is connected to the presence of three His residues in its sequence. Interestingly, PrP 185-208 does not aggregate at pH 5.5, although this peptide possesses one His residue.[9] However, both peptides aggregate in the presence of heparin, and for this reason, heparin was used as an aggregation trigger in all our systems. In the case of PrP 185-208, heparin was necessary to start the process, and in the case of Aβ 1-28, it significantly accelerated fibril formation.[9] The effect of heparin can also be related to the presence of His residues. Both Aβ 1-28 and PrP 185-208 possess binding motives: VHHQKL and VTHQK, respectively.[9] It has been proposed that glycosaminoglycans bind to Aβ peptide to the region 13−16 HHQK that is situated at the interface of two neighboring protofilaments.[27]

There are many reports in the literature showing that the pH optimum for amyloid aggregation is between 5 and 6.[28,29] On the other hand, it is believed that dendrimers require an acidic pH for their maximal inhibiting activity. It has been suggested that the presence of protonated amino groups on the surface of

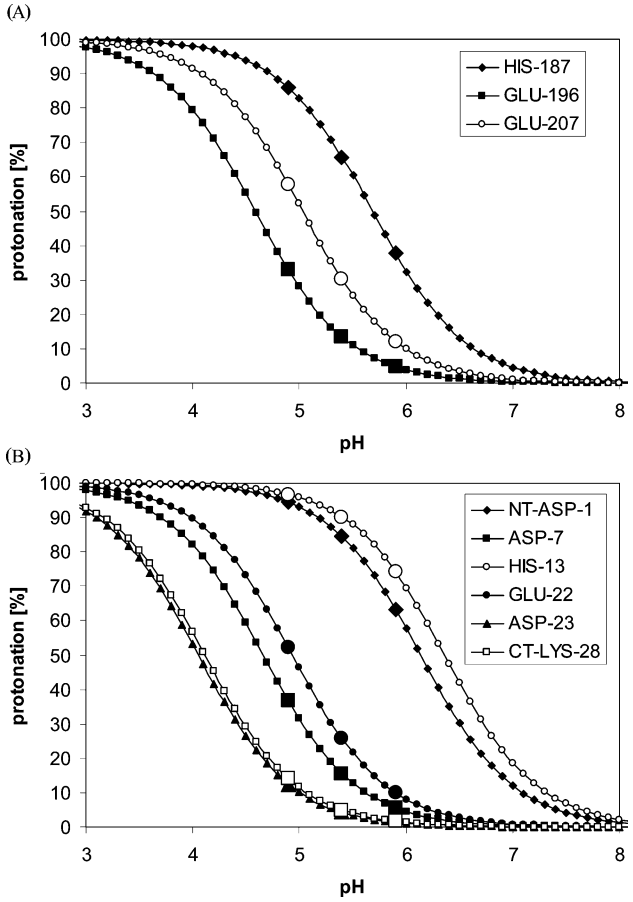

Figure 6. The level of protonation of PrP 185-208 peptide (A) and Aβ 1-28 peptide (B).

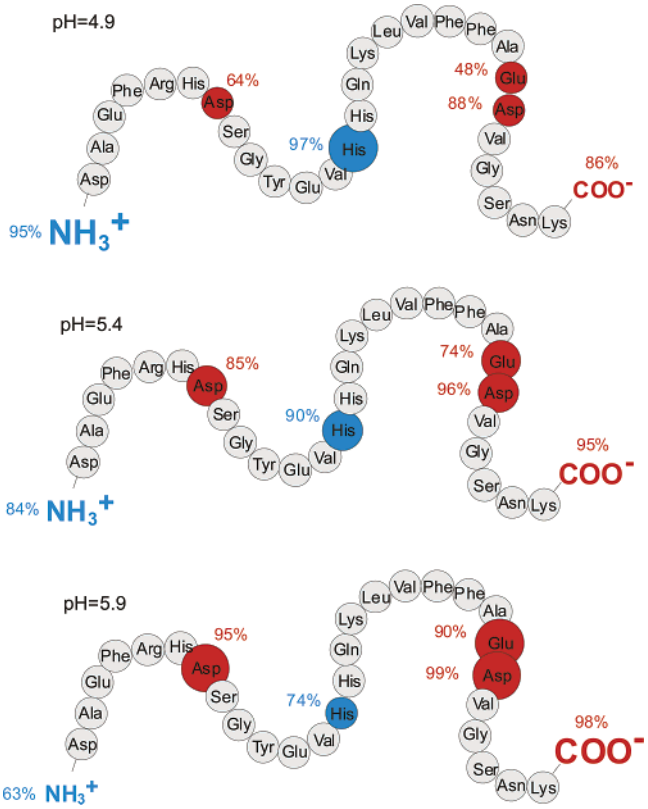

Figure 7. Effect of pH on the charge carried by Aβ 1-28 peptide.

dendrimers is crucial. This was proved by checking that hydroxy-terminated dendrimers were ineffective even at high

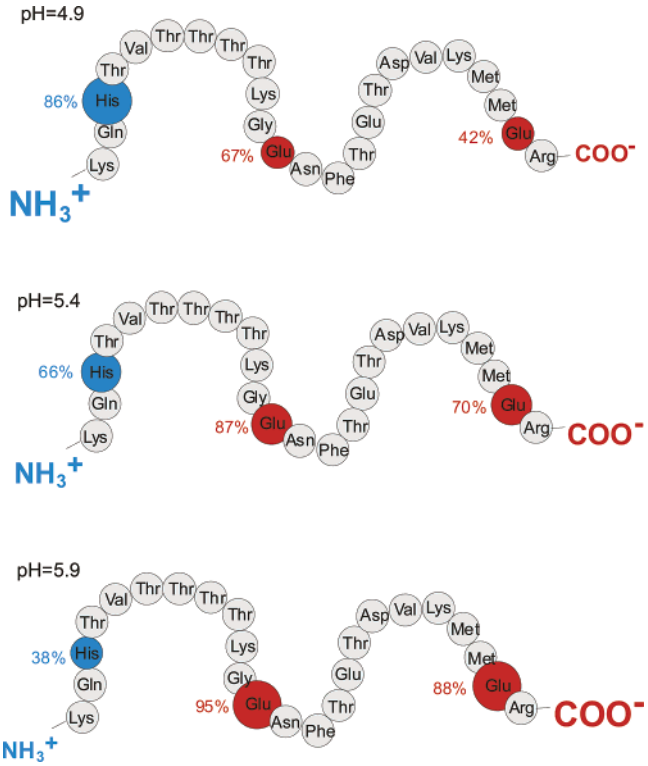

Figure 8. Effect of pH on the charge carried by PrP 185-208 peptide.

concentrations. Moreover, amyloid yield and growth rate decreased when the dendrimer concentration or the dendrimer generation were increased. For PPI dendrimers, this effect reached a plateau for the fourth generation.[30] The number of amino groups can be increased by increasing dendrimer concentration or by using a higher dendrimer generation. However, the number of surface groups is not the only determinant of dendrimer activity. The structure of dendrimers also plays an important role. The concentrations of PAMAM and PPI dendrimers (both possessing 32 amino groups) that are required to reduce PrPSc to 50% of control levels in ScN2 (after cell exposure to dendrimers for 16 h) are 400 and 80 ng/mL, respectively.[30] Probably, both the branching architecture and a high surface density of primary amino groups on the surface are important, although it seems that a more compact structure is favorable.

Cell-based assays where the efficiency of dendrimers was estimated by the decrease in the amount of protease K resistant forms in infected ScN2a cells[8,30] were confirmed by ThT assays.[9,31] However, there are some contradictions in the literature about the mechanism according to which dendrimers act against fibrils. The opinions vary from a purely solubilizing role[32] to a specific binding to the proteins.[8] We have previously postulated a specific binding involving at least two mechanisms: blocking the ends of growing fibrils and increasing the fibril breakage rate.[31] The breakage mechanism is supported by two facts: dendrimers' ability to disrupt pre-existing fibrils and the accelerating effect on fibril formation at low dendrimer concentrations. When the dendrimer concentration is low, it results in a moderate level of fibril breakage. Paradoxically, it can speed up amyloidogenesis by creating new free ends that can serve as sites of replication. This explains why it was previously found that the second generation of PPI dendrimers promoted fibril formation. Low dendrimer generations or low dendrimer concentrations enhance fibril formation.[23] When the dendrimer concentration increases, they can effectively inhibit a fibril formation because the disruption is faster than the production of fibrils. The second inhibiting mechanism—blocking ends—is always concentration-dependent. For both peptides, this process should be more pronounced for higher pH when C-terminal carboxylic acid is ionized.

A recent computational study has proposed that residues 180−193 are one of two fibrillization sites for PrP.[32] The main role is played by His-187. It is known that the aggregated β-sheets are stabilized by long-range effects such as salt bridges. The salt bridge can involve intramolecular interactions between His-187 and Glu-196. These interactions may be disrupted by positively charged dendrimers which have affinity toward the Glu residue. In the case of Aβ 1-28, a salt bridge can be built between Asp-7 and His-13, and again at higher pH, Asp-7 is an attractive target for a cationic dendrimer. However, it seems that, in the case of PrP 185-208 peptide, dendrimers predominantly interact with Glu-207 and Arg-208. These residues are close to the C-terminal side of the peptide; therefore, it favors an end-blocking mechanism.

To summarize, the ionization state of acidic and alkaline residues in prion and Alzheimer's peptides plays an important role in interactions between dendrimers and peptides, which can result in an enhancement or a decrease of the peptidic amyloidogenicity. Our results can help to establish the conditions for which dendrimers are effective inhibitors. These conditions are different for PrP 185-208 and Aβ 1-28 and strongly depend on the amino acid sequence. Our studies show that dendrimers can serve as a tool to study the mechanism of formation of amyloid-like structures and to generate useful knowledge for the design of compounds with therapeutic utility.

Acknowledgment. Study sponsored by a grant 12/IV/2005 from POLPHARMA Foundation for Development of Polish Pharmacy and Medicine.

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BM060229S