# Interactions between dendrimers and heparin and their implications for the anti-prion activity of dendrimers

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Heparin is involved in the pathogenesis of prion diseases, affecting the process of fibril formation. It has been shown that whether it accelerates or inhibits fibrilogenesis depends on its concentration: prion peptide PrP 185-208 aggregates in the presence of 0.04 mg $ml^{-1}$ heparin, but concentrations ten times lower or higher cause no aggregation. Polyamidoamine, polypropyleneimine and phosphorus dendrimers that previously exhibited anti-prion activity have been shown to interact with heparin. The interactions between cationic dendrimers and anionic heparin are mainly electrostatic. The present study shows that these interactions are indirectly responsible for the inhibition or enhancement of fibril formation by dendrimers.



## Introduction

Dendrimers are examples of polymers that are characterized by specific architectures. Each dendrimer consists of a core molecule and layers of branched monomers. The more layers that are attached, the higher the so-called generation (G) of the dendrimer. Therefore, all dendrimers can be viewed as more or less globular structures (mainly depending on the generation number) that contain empty cavities and bear large numbers of functional groups on their surfaces. Dendrimers have recently excited considerable interest; they have been used in many fields, particularly biomedicine. Although more than two decades have passed since the first dendrimers were synthesized,[1] there is still a substantial gap in understanding the nature of their interactions with many medically relevant compounds.

The present study focuses on the interactions between three types of dendrimers and heparin. Heparin is a highly negatively charged glycosaminoglycan (GAG) found in small quantities in many mammalian tissues.[2] It has many medical applications: it is widely used as an anti-coagulant and anti-thrombotic,[3] it inhibits the growth and replication of human immunodeficiency virus (HIV)[4] and it can suppress tumor growth and metastasis by inhibiting angiogenesis.[5] The potential anti-cancer activity of heparin has been supported by *in vitro* studies. However, the development of a non-invasive drug delivery system for heparin has long been an elusive goal.[6] The major barriers hindering delivery include enzymatic degradation, chemical instability and poor absorption across cell membranes. The literature gives examples of attempts to use dendrimers as GAG carriers to facilitate absorption or cellular uptake. Positively-charged polyamidoamine (PAMAM) dendrimers have been shown to be suitable carriers for pulmonary delivery of a low-molecular weight heparin.[7] Heparin–dendrimer aggregates form spontaneously when dendrimers are added to an aqueous heparin solution.[8] Kasai and co-workers synthesized arginine dendrimers that bound strongly to heparin and therefore showed anti-angiogenic activity.[9]

One very interesting aspect is the role of heparin and other polyanionic GAGs in amyloid diseases, which are characterized by the formation of insoluble amyloid fibrils from previously soluble polypeptides. Prion disorders, which are examples of amyloid diseases, are fatal neurodegenerative disorders involving conformational changes from the normal cellular form of prion protein ($PrP^{C}$) to an infectious scrapie isoform ($PrP^{Sc}$). The infectious form is rich in β-structure because the unordered and helical regions in the non-pathological form of the protein are transformed. The β-form leads to the formation of fibrils—amyloid-like structures. Heparin has been found to induce aggregation and β-sheet formation.[10,11] Sulfated GAGs have frequently been identified as potential physiological ligands of $PrP^{C}$, creating oligomeric complexes with PrP.[12] However, there are contradictory hypotheses about the role of GAGs in the infection, since both enhancing and inhibiting effects have been identified.[13,14]

The aims of the present study were: (a) to check—by means of electron paramagnetic resonance (EPR), fluorescence and zeta-potential measurements—the interactions between heparin and dendrimers that have shown to possess anti-prion activity;[15–17] and (b) to analyze by fluorescence measurements the implications of heparin–dendrimer interactions for peptide aggregation. We conducted experiments with dendrimers that

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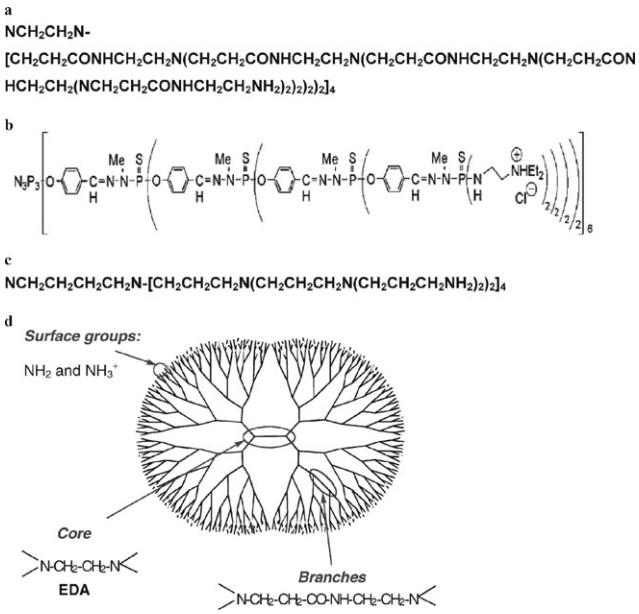


**Fig. 1** Chemical formulas of PAMAM dendrimers G5 (a); P-dendrimers G4 (b); PPI dendrimers G3 (c); bidimensional projection of the structure of PAMAM dendrimer (d).[18]

differed in terms of generation (size) and chemical composition: polypropyleneimine (PPI) dendrimers G3, phosphorus dendrimers (P-dendrimer) G4 and polyamidoamine (PAMAM) dendrimers G5 (Fig. 1). The same set of dendrimers was previously chosen to study interactions with prion and Alzheimer's peptides, further confirming their ability to disturb the process of amyloidogenesis.[18] We used the synthetic peptide PrP 185-208 to follow aggregation in the presence of heparin and dendrimers.

## Experimental

### Materials

Polypropyleneimine (PPI) dendrimers were purchased from SyMO-Chem BV (The Netherlands). Polyamidoamine (PAMAM) dendrimers were obtained from Dendritic NanoTechnologies Inc. (USA). Phosphorus dendrimers (P-dendrimers) were synthesized in the Laboratoire de Chimie de Coordination du CNRS as described previously.[19] All dendrimers were dissolved in aqueous buffers. The synthetic peptide PrP 185-208 [KQHTVTTTTKGENFTETDVKMMER] was purchased from JPT Peptide Technologies GmbH (Germany). Stock peptide solutions were kept in aqueous buffer at pH 7.5. The spin probe 4-trimethylammonium, 2,2,6,6-tetramethylpiperidine-1-oxyl bromide (CAT1) was purchased from Sigma-Aldrich and dissolved in aqueous buffer. Thioflavin T (T-3516) and heparin sodium salt (H-4784) were obtained from Sigma Chemical Company. All other chemicals were of analytical grade. Double-distilled water was used to prepare all solutions.

### EPR spectroscopy

EPR spectra of CAT1 (Fig. 2) (0.5 mM) were recorded with an EMX-Bruker spectrometer operating at the X band (9.5 GHz)

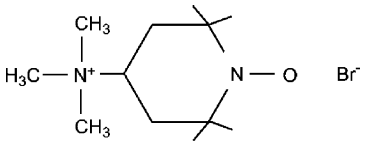


**Fig. 2** The structure of CAT-1.

and interfaced with a PC (software from Bruker for handling and analyzing the EPR spectra). The temperature was controlled with a Bruker ST3000 variable-temperature assembly cooled with liquid nitrogen.

Because these techniques differ in sensitivity, higher concentrations were used in the EPR experiments than in the fluorescence experiments, but the heparin–dendrimer ratio was in the same range in both cases (1–40000 g $l^{-1}$ of heparin per 1 mol of dendrimer).

The EPR spectra were subjected to computer-aided analysis by the well-established procedure reported by Budil and co-workers.[20] The spectra of the free probe in solution at room temperature comprised three hyperfine (hp) lines ($2I_N + 1 = 3$ lines). The main input parameters were as follows. (a) The $g_{ii}$ components (for the coupling between the electron spin and the magnetic field) were the ones previously used for the nitroxide ($g_{xx} = 2.009$, $g_{yy} = 2.006$, $g_{zz} = 2.0025$)[18] and were considered constant for all samples. (b) The $A_{ii}$ principal values of the **A** tensor for the coupling between electron and nuclear spin. An increase in the environmental polarity of the NO group provokes an increase in the **A** tensor components owing to the increased electron spin density on the nitrogen nucleus. (c) The perpendicular component of the correlation time for rotational diffusion ($\tau_{perp}$). Brownian motion was assumed in the calculation, for which the diffusion component is $D_{perp} = 1/(6\tau_{perp})$.

When the probes aggregate, the spin–spin interactions among them provoke line broadening and the eventual collapse of the three lines. In this event, the computation needs the following input parameters: (i) the exchange frequency, $\omega_{ex}$, due to radicals colliding (in a fluid system) because of their high local concentration; (ii) the intrinsic line width that increases with the increase in dipole–dipole interactions due to increased radical proximity.

To extract each of the components that were superimposed in the experimental spectrum, we subtracted experimental spectra that contained the same components but at different relative intensities.

### Zeta-potential measurements

Zeta-potential experiments were carried out on a Malvern Instruments Zetasizer 2000 (UK) using a standard rectangular quartz cell. Samples were dissolved in 10 mM phosphate buffered saline (pH 7.5 and 5.5) and measurements were performed at room temperature.

### Thioflavin T (ThT) assay

Amyloid fibrils can be produced *in vitro* by exposing disease-associated peptides such as prion peptide PrP 185-208 to destabilizing conditions (adding heparin and lowering the pH). The aggregation process was monitored using the dye

 

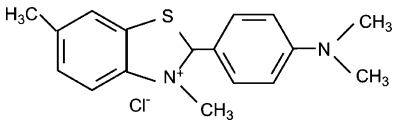


**Fig. 3** The structure of ThT.

thioflavin T (ThT) (Fig. 3) that fluoresces when amyloid structures are present. A stock solution of peptide (1.2 mM) in Tris buffer (pH 7.5) was diluted to a final concentration of 50 μmol $l^{-1}$. ThT was then added (final concentration 35 μM) followed by heparin (range of concentrations tested = 0.004–0.4 mg $ml^{-1}$), and the pH was adjusted to 5.5 with HCl (stock solution 0.01 M). Fluorescence measurements were performed at 37 °C with continuous stirring using a Perkin-Elmer LS-50B spectrofluorimeter. Experiments were performed in the absence and presence of dendrimers. The kinetics of aggregation were monitored by recording fluorescence intensity. The excitation and emission wavelengths were 450 and 490 nm, respectively. The excitation and emission slit widths were set to 5 nm.

The fluorescent dye ThT was also used to monitor interactions between P-dendrimers and heparin. Increasing concentrations of heparin were added to a solution containing 1 μM or 0.1 μM P-dendrimer and the fluorescence intensity was recorded. The excitation and emission wavelengths were 450 and 482 nm, respectively. The excitation and emission slit widths were set to 5 nm. Experiments were done at pH 5.5 and 7.5.

## Results and discussion

### Interactions between dendrimers and heparin

**EPR studies.** When increasing concentrations (0 to 1 mg $ml^{-1}$) of heparin were added to 0.5 mM CAT1, the EPR spectra at 255 K showed interesting behavior. The intensity of the free component increased relative to the aggregated component characteristic of pure CAT1 solutions up to heparin concentrations of 0.4 mg $ml^{-1}$ (about 0.06 mM). At that concentration the EPR spectrum showed only the free component. However, the free component almost disappeared at 0.6 mg $ml^{-1}$ heparin. Fig. 4a illustrates these changes in the EPR spectra of CAT1 at heparin concentrations of 0.2, 0.4 and 0.6 mg $ml^{-1}$. At 0.2 mg $ml^{-1}$ the spectrum shows two superimposed components: (a) a single line broad signal ascribed to CAT1 aggregates (salt separation); and (b) a three narrow line signal attributed to a small fraction of probes that were mobile in the hydration layer of heparin. At 0.4 mg $ml^{-1}$, almost all the CAT1 is extracted from the aggregates and distributed into the heparin hydration layer due to the electrostatic attraction between the positively charged probe and the negatively charged heparin sites. This interaction is not so strong as to induce slow motion of the probe at room temperature, but the probe moves fast in the hydration layer of heparin, in spite of the low temperature, due to the change in the rheological properties of water at the water–heparin interphase. However, at concentration 0.6 mg $mL^{-1}$ of heparin, probably the very high charge of heparin and the competition of CAT1 with the counter-ions (described in the review by Rabenstein)[2] provoke the condensation of CAT1. Over the dendrimer concentration range 5–20 μM, the dendrimer–CAT1 system gave rise to CAT1 aggregation, so only a single broad line signal was recorded. Therefore, the two systems, heparin (0.4 mg $ml^{-1}$) + CAT1 and dendrimer (5–20 μM) + CAT1, provided two completely different EPR responses: only the free component and only the aggregated component, respectively.

When dendrimers (5–20 mM) and heparin (0.4 mg $ml^{-1}$) were mixed in solution, both the aggregated and the free CAT1 components contributed to the spectra; but, interestingly, an interacting component also appeared. For P-dendrimer, the intensity of this interacting component increased with increasing dendrimer concentration from 5 to 20 μM. Therefore, as shown in Fig. 4b, the three components—free, aggregated and interacting—were readily recognizable (indicated by arrows) in the P-dendrimer (20 μM) + heparin (0.4 mg $ml^{-1}$) spectrum. These components were extracted by the subtraction procedure described above (the subtraction and double integration of the components provided the percentages of the components themselves: 68% aggregated, 25% interacting, 7% free) and then computed. Fig. 4b also shows the experimental and computed components and the main parameters used for computation. The interacting component is not present in the "only heparin" or "only dendrimer" systems; it appears when the dendrimer and heparin are mixed. Therefore, it reports on heparin–dendrimer interactions that mainly occur with P-dendrimers. The relatively low $A_{ii}$ components, listed in Fig. 4b for this interacting component, indicate that a low polarity region hosts the probes.

Fig. 4c shows the experimental and computed spectra of the free component of CAT1 in heparin solution at the same concentration. The mobility of the CAT1–heparin system is much higher than it is when both P-dendrimer (20 μM) and heparin (0.4 mg $ml^{-1}$) are present.

We noted that both the relative intensity and $\tau_{perp}$ of the free component changed from one system to another. These parameters, reported in Table 1, are modified by (a) the heparin concentration, (b) the dendrimer concentration and (c) the type of dendrimer. Of course, the temperature also affects this ratio, but the maximum information about the differences among the various systems is obtained at 255 K. Analysis of the EPR data suggests the following:

In the presence of dendrimers, as in their absence, the relative intensity of the free component is higher at 0.4 mg $ml^{-1}$ heparin than at 0.2 and 0.6 mg $ml^{-1}$ and is lowest at 0.6 mg $ml^{-1}$. This is in line with the two effects described above: (a) disaggregation of the probes due to their insertion in the hydration layer of heparin when the heparin concentration is increased from 0.2 to 0.4 mg $ml^{-1}$; (b) aggregation of the probes when heparin concentration is 0.6 mg $mL^{-1}$, due to a competition among the charged interacting groups and ions.[2]

For the free component, the higher the relative intensity, the higher the mobility, because more of the probe partitions into the hydration layer of heparin. However, the mobility is lower in all cases than it is in pure heparin solutions, indicating that the hydration layer becomes a dendrimer–heparin interphase.

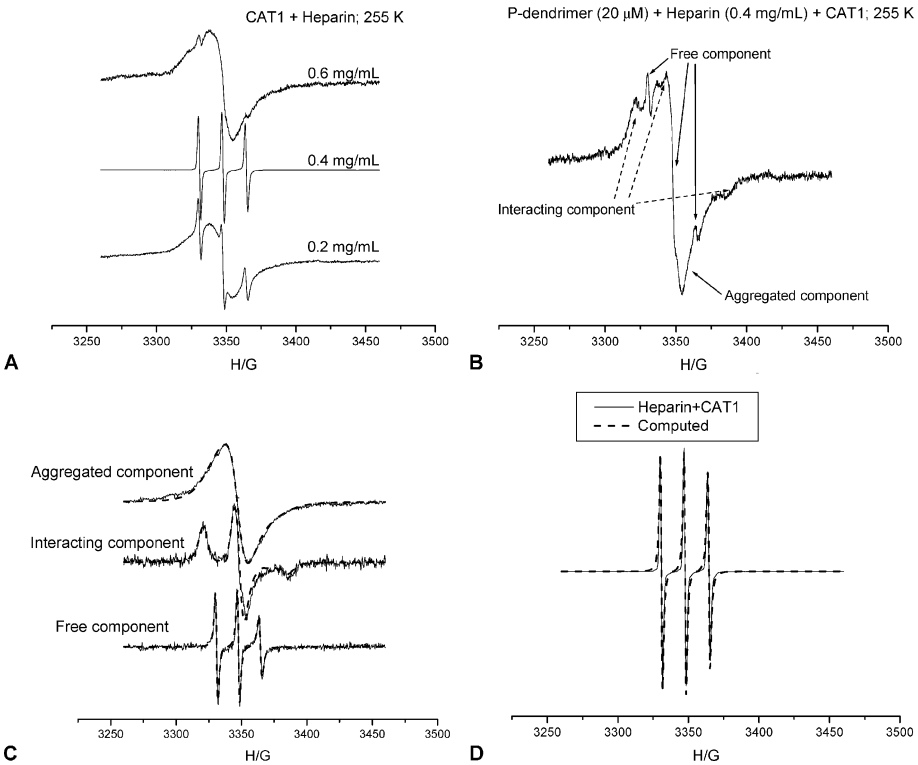


**Fig. 4** (A) EPR experimental spectra (255 K) of CAT1 (0.5 mM) at heparin concentrations of 0.2, 0.4 and 0.6 mg $mL^{-1}$. (B) EPR spectrum of P-dendrimer (20 μM) and heparin (0.4 mg $mL^{-1}$) in water solution at 255 K. The three components—free, aggregated and interacting—are indicated with arrows. (C) Experimental subtracted components, and their computation (dashed lines) for the interacting, aggregated and free probes. The main parameters used for computation are: aggregated component: line width = 8 G, $\omega_{ex} = 3 \times 10^8$ $s^{-1}$; interacting component: $A_{ii}$ = 5 G, 4 G, 34 G, $\tau_{perp} = 1 \times 10^{-8}$ s; free component: $\tau_{perp} = 3.8 \times 10^{-10}$ s. (D) Experimental and computed free component ($\tau_{perp} = 1 \times 10^{-10}$ s) of CAT1 in the heparin solution at the same concentration.

**Table 1** Changes in relative intensity and $\tau_{perp}$ for a free component

| Type of dendrimer | Dendrimer concentration/μM | Heparin concentration/mg $mL^{-1}$ | Relative intensity free component/A.U. | $\tau_{perp}$ free component/$10^{-10}$ s |
|---|---|---|---|---|
| PAMAM | 10 | 0.4 | 146 | 2.3 |
| PAMAM | 20 | 0.4 | 6 | 3.6 |
| PAMAM | 10 | 0.2 | 20 | 3.65 |
| PAMAM | 10 | 0.6 | 5 | 4.2 |
| PPI | 10 | 0.4 | 1000 | 1.2 |
| PPI | 20 | 0.4 | 10 | 4.05 |
| PPI | 10 | 0.2 | 8.5 | 3.65 |
| PPI | 10 | 0.6 | 4.5 | 3.75 |
| P-dendrimer | 10 | 0.4 | 33.5 | 3.65 |
| P-dendrimer | 20 | 0.4 | 20.5 | 4.2 |
| P-dendrimer | 10 | 0.2 | 18 | 4.15 |
| P-dendrimer | 10 | 0.6 | 19 | 4.3 |

We have to take into account that CAT1 positive charge is neutralized by the negatively charged heparin sites and therefore it is not repulsed by the positively charged amino groups at the dendrimer surface.

P-dendrimers interact more strongly with heparin and show less variation with experimental conditions. Only a small fraction of the probe remains free in the dendrimer–heparin interphase, with relatively slow mobility and showing little change in response to differences in heparin and dendrimer concentrations.

PAMAM and especially PPI dendrimers show significant variation in the amount and mobility of the free component, depending on both the dendrimer and the heparin concentrations. Very little free probe remains at the highest dendrimer and heparin concentrations, and its mobility is relatively slow. However, at 10 μM PPI and 0.6 mg $ml^{-1}$ heparin, the spectrum indicates PPI–heparin interactions, though to a lesser extent than P-dendrimer–heparin interactions.

**Zeta-potential.** The ability to create heparin–dendrimer complexes was further confirmed by zeta-potential experiments (Fig. 5). The zeta-potential of the heparin–dendrimer complex decreased until the concentration of heparin reached 0.04–0.06 mg $ml^{-1}$; no changes were observed at higher

 

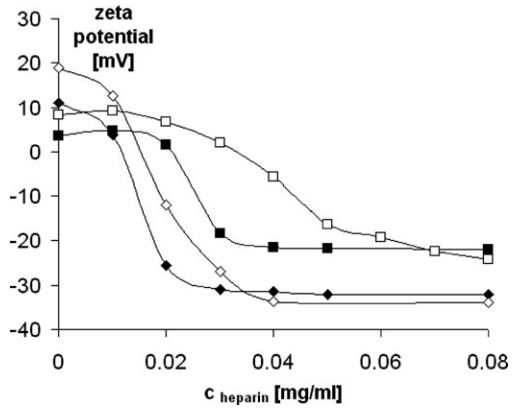


**Fig. 5** Changes in zeta-potential upon addition of heparin to the solution containing: 1 μM of PAMAM dendrimers at pH 5.5 (open square), at pH 7.5 (filled square); 1 μM of P-dendrimers at pH 5.5 (open rhombus), at pH 7.5 (filled rhombus).

concentrations. The final zeta-potential values (−20 to −30 mV) show that the complexes are relatively stable. For both PAMAM and P-dendrimers the plateau was reached at lower heparin concentration when the pH of the sample was 7.5 (compared to samples with pH 5.5). At lower pH, more molecules of heparin attached to one dendrimer molecule. This demonstrates that the heparin–dendrimer interaction is electrostatic. The sigmoidal shape of the zeta-potential curve suggests the presence of multiple binding sites on the dendrimer surface, consistent with the aggregation of CAT1 at the dendrimer surface as monitored by EPR.

**Fluorescence studies.** A very interesting phenomenon was observed with the P-dendrimer–heparin complexes in the presence of the fluorescent ThT probe: although ThT did not fluoresce in the presence of dendrimers alone or heparin alone, a fluorescence spectrum was recorded for the P-dendrimer–heparin complex. Only the P-dendrimer behaved in this way. The dendrimer–ThT solution was titrated with heparin and the fluorescence intensity of ThT increased (Fig. 6a). Similarly, the spectrofluorimetric titration of the heparin–ThT solution with dendrimer produced an increase in ThT fluorescence intensity (Fig. 6b). The effect was more pronounced under acidic conditions when amino groups of dendrimers were ionized, further supporting the hypothesis that the dendrimer–heparin interaction is electrostatic. ThT emits fluorescence when it is immobilized and its fluorescence selectively depends on steric constraint of the rotation of the ThT ring. Excitation results in intramolecular charge transfer between adjacent ThT rings. This charge shift can be stabilized if one of the rings rotates by 90°, making the reverse shift impossible.[21] This condition is met when ThT binds to the fibrils with its long axis parallel to them,[22] but not when ThT is in the presence of amorphous protein aggregates. Fibrils are the most regular naturally-occurring protein aggregates. This implies that phosphorus dendrimers and heparin must create a kind of net that incorporates ThT molecules much as fibrils do. Imae and co-workers, analyzing the interactions of PAMAM dendrimers and sodium hyaluronate, described several models for the interactions between these compounds, starting from a non-binding model, through an average binding model and a

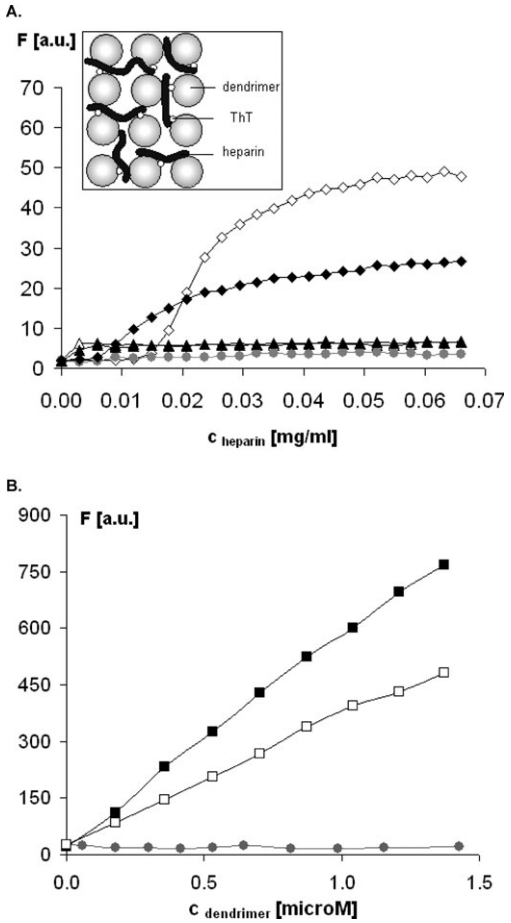


**Fig. 6** (A) ThT fluorescence variations upon addition of heparin to the solution containing: 35 μM of ThT (filled circle); 35 μM of ThT and P-dendrimer at concentration 0.1 μM at pH 5.5 (open triangle) and pH 7.5 (filled triangle); 35 μM of ThT and P-dendrimer at concentration 1 μM at pH 5.5 (open rhombus) and pH 7.5 (filled rhombus). (B) ThT fluorescence variations upon addition of phosphorus dendrimers to the sample containing ThT at concentration of 35 μM (gray circles), ThT (35 μM) and heparin at concentration 0.04 mg $ml^{-1}$ at pH 5.5 (filled squares), ThT (35 μM) and heparin at concentration 0.04 mg $ml^{-1}$ at pH 7.5 (open squares).

dimeric polymer model to a critical binding model.[23] The dimeric polymer model assumed that sodium hyaluronate formed dimers conjugated by dendrimers. It is likely that a similar situation occurs in the presence of P-dendrimers and heparin and that a complex, regular system is created (Fig. 6a, inset).

In contrast to P-dendrimers, PAMAM dendrimers and PPI dendrimers mixed with heparin did not affect the fluorescent properties of ThT. The distinctive behavior of the P-dendrimers is in agreement with the EPR results, where P-dendrimers caused the biggest changes in spectra.

### Anti-prion implications of interactions between dendrimers and heparin

The importance of GAGs in the aggregation of prions has long been studied, though controversies remain since both protective and enhancing effects of GAGs have been described. Besides animal and cell models, an experimental

model using parts of the prion protein may be used to study the aggregation process. These prion peptides can create fibrils when they are exposed to destabilizing factors. In the case of prion peptide PrP 185-208, aggregation was achieved by lowering the pH to 5.5 and adding heparin.[24] The accumulation of amyloids can be monitored by changes in ThT fluorescence, which is sensitive to the presence of amyloid fibrils.[22] This allows the kinetics of the process to be monitored and gives additional information about inhibitors.[25] As we showed earlier, the presence of 0.04 mg $ml^{-1}$ heparin was enough to start the aggregation of 50 μmol $l^{-1}$ PrP 185-208.[24] In the present study we broadened the concentration range and used ThT fluorescence to test whether aggregation occurs in the presence of 0.004, 0.02, 0.04 and 0.4 mg $ml^{-1}$ heparin (Fig. 7a). No aggregation was observed at the lowest heparin concentration. This can be explained by the hypothesis that heparin acts like a nucleation seed. When the concentration of heparin is too low, no triggering effect is seen. However, the mechanism of action must be far more complicated since too high a heparin concentration completely inhibited fibril formation. Moreover, 0.02 mg $ml^{-1}$ heparin caused faster aggregation than 0.04 mg $ml^{-1}$. This means that the effect of heparin is a result of two opposing processes: acceleration of fibril formation up to a certain concentration and inhibition above that concentration. This partially explains the controversies in the literature about the role of heparin.

PAMAM, PPI and P-dendrimers have been shown to affect the kinetics of fibril formation. All kinetic experiments for PrP 185-208 were performed in the presence of heparin.[15–17] On the other hand, as shown above, all these dendrimers interact with heparin. This raises the question of whether heparin–dendrimer interactions play an important role during fibril formation in the presence of dendrimers. It is worth stressing that a common phenomenon observed for all dendrimers was not linearly concentration-dependent behavior. It means that dendrimers enhanced fibril formation at low concentrations and inhibited the process at high concentrations. To determine the role of heparin, kinetic experiments were performed with 0.02, 0.04 and 0.4 mg $ml^{-1}$ heparin in the presence of dendrimers. The results described below were analogous for all dendrimers tested. For clarity they are only shown for PAMAM dendrimers. When the PAMAM dendrimer concentration was increased in the presence of 0.04 mg $ml^{-1}$ heparin, fibril formation was accelerated at $C_{PAMAM}$ = 0.01 μM (Fig. 7b); the process was significantly inhibited at $C_{PAMAM}$ = 0.1 μM (Fig. 7c); finally, fibril formation was completely inhibited at $C_{PAMAM}$ = 1 μM (Fig. 7d).

Besides these results, other interesting facts were observed. PAMAM dendrimers at 0.01 μM promoted fibril formation when 0.04 mg $ml^{-1}$ heparin was present, but they significantly inhibited aggregation at 0.02 mg $ml^{-1}$ heparin. PAMAM dendrimers at 0.1 μM reduced fibril formation to some extent in the presence of 0.04 mg $ml^{-1}$ heparin, but they completely stopped the process in the presence of 0.02 mg $ml^{-1}$ heparin.

These observations can be explained if we assume that dendrimers and PrP compete for interaction with heparin. By interacting with heparin, dendrimers reduce the amount that is free to interact with PrP. Therefore, when 0.01 μM PAMAM dendrimer is present (Fig. 7b) and when 0.04 mg $ml^{-1}$ heparin is added, some of the heparin is absorbed by the dendrimers and the actual heparin concentration that affects fibril formation is less than 0.04 mg $ml^{-1}$. The overall effect therefore resembles the aggregation process in the absence of dendrimers when the heparin concentration

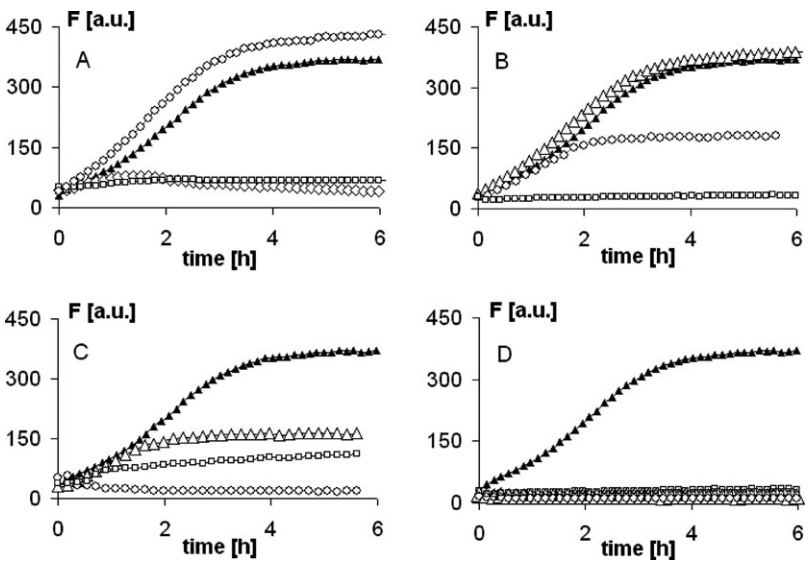


**Fig. 7** Changes in ThT fluorescence during the aggregation process of PrP 185-208 (A) in the absence of dendrimers and in the presence of heparin at concentration: 0.02 mg $ml^{-1}$ (open circle); 0.04 mg $ml^{-1}$ (filled triangle); 0.4 mg $ml^{-1}$ (open square); 0.004 mg $ml^{-1}$ (open rhombus); (B) in the presence of 0.01 μM PAMAM dendrimers and in the presence of heparin at concentration: 0.02 mg $ml^{-1}$ (open circle); 0.04 mg $ml^{-1}$ (open triangle); 0.4 mg $ml^{-1}$ (open square); control (no dendrimers, heparin at concentration 0.04 mg $ml^{-1}$) (filled triangle); (C) in the presence of 0.1 μM PAMAM dendrimers and in the presence of heparin at concentration: 0.02 mg $ml^{-1}$ (open circle); 0.04 mg $ml^{-1}$ (open triangle); 0.4 mg $ml^{-1}$ (open square); control (no dendrimers, heparin at concentration 0.04 mg $ml^{-1}$) (filled triangle); (D) in the presence of 1 μM PAMAM dendrimers and in the presence of heparin at concentration: 0.02 mg $ml^{-1}$ (open circle); 0.04 mg $ml^{-1}$ (open triangle); 0.4 mg $ml^{-1}$ (open square); control (no dendrimers, heparin at concentration 0.04 mg $ml^{-1}$) (filled triangle).

 

is 0.02 mg $ml^{-1}$ (Fig. 7a). For PAMAM dendrimers at 0.1 μM we observed stronger inhibition when the heparin concentration was halved, because hardly any free heparin remained to interact with the PrP.

This hypothesis was further supported when a high, inhibiting concentration of heparin was used (0.4 mg $ml^{-1}$). At this concentration, no aggregation occurred in the system without dendrimers (Fig. 7a). Adding dendrimers at low concentration did not significantly change the situation (Fig. 7b), but when a ten-fold higher concentration of dendrimers was applied, a slow aggregation process started (Fig. 7c). This means that dendrimers at 0.1 μM started to become efficient in reducing the amount of heparin available for PrP binding.

Dendrimers are not the only anti-prion compounds that interact with heparin. Copaxone, an immunomodulatory agent used to treat multiple sclerosis, has been shown to affect the initial prion infection process. It has been proposed that Copaxone delays prion infection by competing with $PrP^{Sc}$–glucosaminoglycan interactions.[26] Such competition is possible because Copaxone can bind heparin. Zsila and Gedeon tested several cationic anti-prion agents with unknown mechanisms of action and they all turned out to interact with heparin.[27]

The picture would not be complete if we did not point out our final observation: when the highest concentration of dendrimers was applied (1 μM), no fibril formation was observed no matter how much heparin was in the system. It is unlikely that the highest concentrations of heparin were completely purged from the system in this case.

## Conclusions

Interactions between dendrimers and heparin are indirectly responsible for the inhibition or enhancement of fibril formation by dendrimers. It happens because dendrimers electrostatically interact with heparin that decreases the amount of heparin, which can trigger the amyloidogenesis. However, one should remember that even though "the heparin factor" explains the behavior of dendrimers very well, it is not the only factor that should be taken into account when investigating the mode of dendrimer action. It has been shown that dendrimers interact with PrP[18] and that they can disrupt existing aggregates.[15,16] At high concentrations, dendrimers act directly to impede fibril formation, whereas at low concentrations they indirectly impede fibril formation by sequestering the heparin from PrP.

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