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Binding of poly(amidoamine), carbosilane, phosphorus and hybrid dendrimers to thrombin—Constants and mechanisms

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ABSTRACT

Thrombin is an essential part of the blood coagulation system; it is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin, and catalyzes many other coagulation-related reactions. Absorption at its surface of small nanoparticles can completely change the biological properties of thrombin. We have analyzed the influence on thrombin of 3 different kinds of small nanoparticles: dendrimers (phosphorus-based, carbosilane based and polyamidoamine) and 2 hybrid systems containing carbosilane, viologen and phosphorus dendritic scaffolds in one single molecule, bearing different flexibility, size and surface charge. There was significant alteration in the rigidity of the rigid dendrimers in contrast to flexible dendrimers. These differences in their action are important in understanding interactions taking place at a bio-nanointerface.

1. Introduction

Dendrimers are a new class of nanotechnological polymers with well-defined molecular structures suitable for targeting, microarray systems, catalysis and drug delivery systems. These approaches are based on the injection of dendrimer-based drugs or agents into the blood stream [1–6]. Thrombin is an essential part of the blood coagulation system; it is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions [7]. To date, some experiments have been made on the interaction between proteins and dendrimers that have indicated the role of the protein structure in these interactions [8–10]. Small nanoparticles can also form a 'nanoparticle corona' with proteins, with at least 3 different types of interactions seen depending on the nature of the protein. First, this corona has no effect on proteins with rigid structures and active sites buried deep within them. Second, it affects the structure of flexible proteins without changing their enzyme activity in cases where the active site is deeply buried. Third, it changes both structure and enzyme activity of flexible proteins that have surface-based active centers [8–10].

To explore the role of nanoparticle flexibility in the 'dendrimer-protein' interactions, we obtained circular dichroism spectra of thrombin in the presence of nanoparticles and their effect on thrombin fluorescence. Circular dichroism shows the secondary structure of proteins, whereas fluorescence quenching monitors changes in protein conformation after addition of nanoparticles [8–10].

2. Materials and methods

Human thrombin, cationic poly(amidoamine) dendrimers of 3rd and 4th generations (PAMAM-NH2 g3 and PAMAM-NH2 g4),

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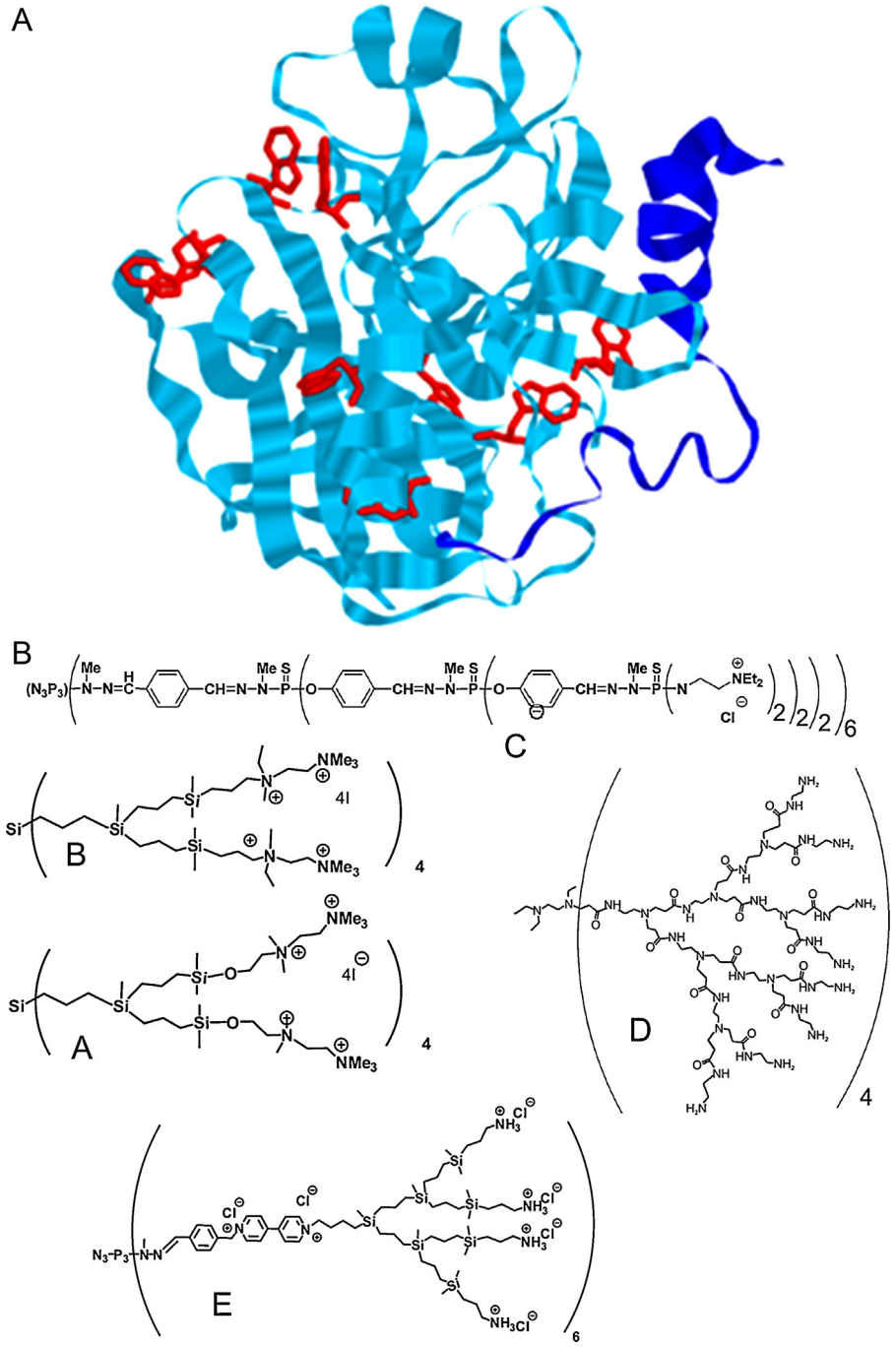

Fig. 1. Structure of α-thrombin from human blood (small subunit – 36 AA – is marked as dark blue, big subunit – 259 AA – is marked as blue, 9 tryptophanyls are marked as red). Dendrimers: A – CBD-OS (2G-NN16), B – CBD-CS, C – phosphorous CPD g3, D – PAMAM g3, E – hybrid SMT2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and sodium phosphate buffer were purchased from Sigma-Aldrich (USA). Other dendrimers had previously been synthesized by us and are described in detail in the following reerences [1–3,11–14]. The buffer was passed through a 0.22-μm filter to remove trace particles. Complexes for the experiments were prepared in PBS by mixing samples at different molar ratios for 15 min in a Vortex mixer.

2.1. Transmission electron microscopy

A copper grid of 200 grid mesh precoated with carbon was glow discharged. A sample in solution was dropped on to the grids, followed by absorbing the excess liquid with filter paper before they were air-dried under a heat lamp. They were stained with 2% uranyl acetate to enhance the contrast between the aggregates and the background before being examined in a Jeol JEM-1010 (Japan) electron microscope.

2.2. Circular dichroism

Measurements were made in 10 mmol/l sodium phosphate buffer (pH 7.4) at 25 °C. Thrombin at different concentrations was incubated with corresponding aliquots of dendrimers (stock solutions in 10 mmol/l sodium phosphate buffer, pH 7.4) for 10 min

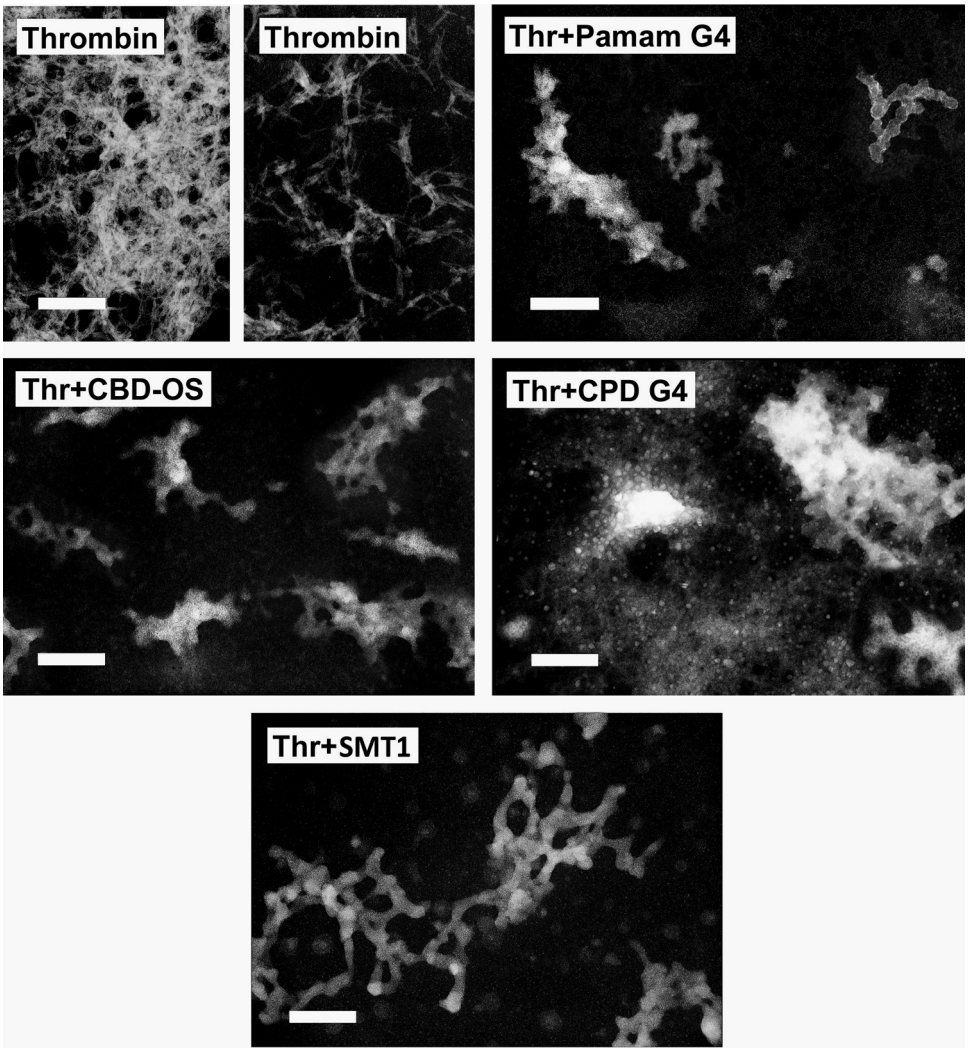

Fig. 2. Transmission electron microscopy images of thrombin in the absence and presence of PAMAM g4, carbosilane (CBD-OS), phosphorus (CPD g4) and hybrid (SMT1) dendrimers. Bar – 100 nm.

at 25 °C. To solutions of thrombin 0–25 μmol/l of dendrimers were added and mixed continuously for 15 min. The CD spectra of thrombin and its complexes with dendrimers were measured with a Jasco-815 spectropolarimeter (Jasco, Japan). Pure dendrimers had no L-R absorption differences under our conditions (i.e. they were not chiral). All spectra were corrected using a baseline obtained with a dendrimer sample, and were smoothed using a binomial algorithm provided by Jasco. Scans were taken from 320 to 200 nm at a rate of 50 nm min^{-1} with a bandwidth of 1 nm in quartz cuvettes and a path-length of 0.2 cm.

2.3. Fluorescence quenching

Thrombin was dissolved in 10 mmol/l sodium phosphate buffer (pH 7.4) at 0.5 μmol/l. Increasing aliquots (0–25 μmol/l) of dendrimer were added to a protein from a stock solution in 10 mmol/l sodium phosphate buffer (pH 7.4) and mixed continuously for 15 min. The fluorescence emission intensity (F) at λ = 350 nm was measured at 25 °C after excitation at 295 nm with a Perkin-Elmer LS-55 spectrofluorimeter (Perkin-Elmer, USA). The emission slit width was kept at 5 nm as was excitation. Fluorescence intensities were normalized as F/F0, where F is the intensity of thrombin fluorescence in presence of a dendrimer and F0 is that of the pure protein. PAMAM g3 and g4 dendrimers had no fluorescence under our conditions.

2.4. Statistical analysis

The data are expressed as mean ± S.D. of 6 independent experiments. Significance of the differences was assessed using the one-way analysis of variance with the post-hoc Newman-Keuls multiple comparisons test.

3. Results

3.1. Thrombin and dendrimers

Fig. 1 shows the structures of thrombin and dendrimers. Thrombin consists of 2 subunits: big and small, and 9 tryptophanyls. Four types of cationic dendrimers were chosen which are described in details in the following references [1–3,11–14]. Dendrimers differed in: i) internal structure: PAMAM dendrimers of 3rd (PAMAM g3) and 4th (PAMAM g4) generations are very flexible and partly hydrophilic inside [1,4], whereas phosphorus dendrimers of 3rd (CPD g3) and 4th (CPD g4) generations are hydrophobic and more rigid [3,11]. The carbosilane dendrimers of 2nd generation bearing –O-Si- (CBD-OS or 2G-NN16) or –C-Si- groups (CBD-CS) are

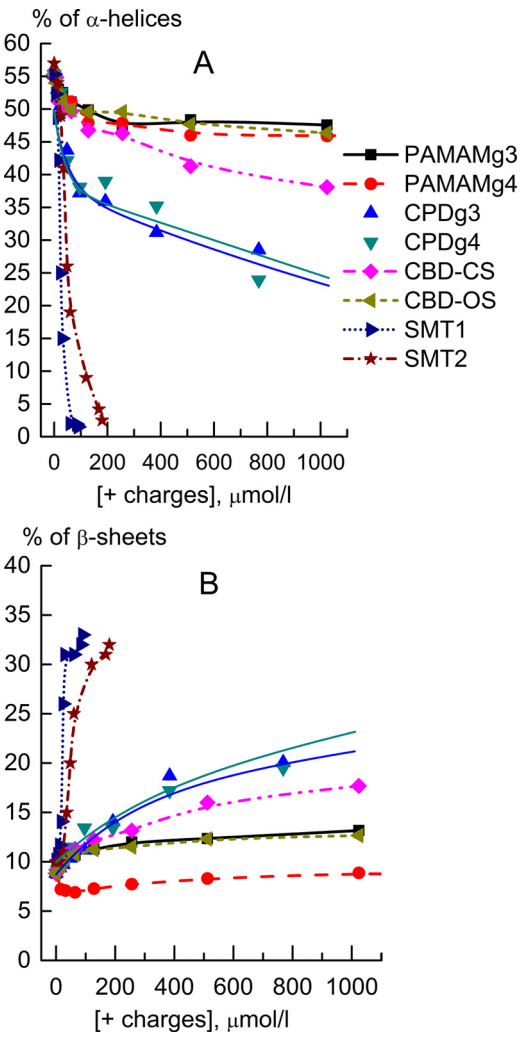

Fig. 3. Changes in thrombin secondary structure after addition of 0–25 μmol/l of dendrimers (expressed in dendrimer positive charges).

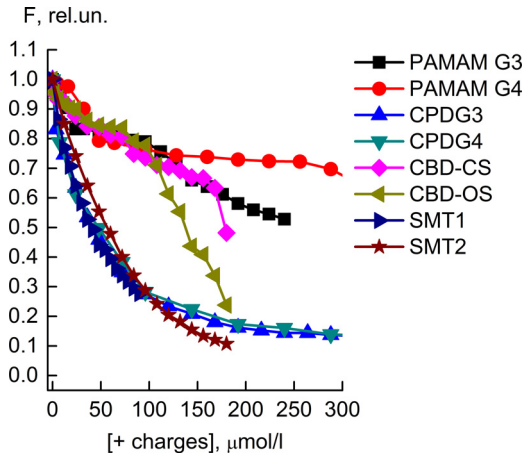

Fig. 4. Changes in thrombin conformation (reflected by fluorescence quenching) after addition of 0–25 μmol/l of dendrimers (expressed in dendrimer positive charges). $\lambda_{ex.}$ = 295 nm, $\lambda_{em.}$ = 350 nm.

hydrophobic as CPD, but are flexible as PAMAM dendrimers [12]. ii) surface charge: phosphorus dendrimers are the biggest and the most charged dendrimers, with 48 (g3) and 96 (g4) positive charges, with a diameter of 6–8 nm [3,11], whereas PAMAM g3 and g4 have 32 (g3) and 64 (g4) positive charges and a 4 nm diameter [1,4]; carbosilane dendrimers of 2nd generation (NN16 and BDBR11) with 24 positive groups have the smallest diameter (~3 nm) [12]; iii) the nature of surface cationic groups: PAMAMs have $-NH_3^+$ groups with protonation being pH-dependent [14]. Phosphorus dendrimer has 'mono-protonated' ammonium groups (-$NH(Et_2)_2$),and CBD has fully alkylated groups (pH independent) [11–13]. SMT1 and SMT2 dendrimers are hybrid systems containing carbosilane, viologen and phosphorus dendritic scaffolds in one single molecule as examples of an "onion peel" strategy. Starting from the hexafunctionalized rigid phosphorus core, viologen units (responsible for polarity) were incorporated into the branching points, decorating their surface by inclusion of cationic carbosilane dendrons (responsible for lipophilicity). These dendrimers contain 2 kinds of cations formed by viologen quaternized units located at inner branches and ammonium groups at the surface of the carbosilane wedges. The inner charge is 12 for both dendrimers, whereas the outer charge is 12 and 24 for SMT1 and SMT2, respectively [13].

3.2. Transmission electron microscopy

Fig. 2 shows the images of thrombin in the absence and presence of PAMAM g3, carbosilane (CBD-OS), phosphorus (CPD g4) and hybrid (SMT1) dendrimers (the data from other dendrimers are not presented here). Dendrimers significantly affected the structure of thrombin. Native thrombin in a dry state has uniformly thin fibrils. Addition of dendrimers led to the formation of big fibrils and aggregates of thrombin in a dry state.

3.3. Circular dichroism (protein secondary structure)

Adding 0–25 μmol/l of dendrimers led to concentration-dependent changes in the secondary structure of thrombin (Fig. 3), depending significantly on the nature of the nanoparticle. The bigger generation of a dendrimer, the higher is its positive charge [1–3,11–14]. Big dendrimers have a significant amount of positive charges at their surface that interact with negatively charged amino acids residues of the proteins [8–10]. To find another interactions except electrostatic ones between dendrimers and thrombin [8–10], we expressed the concentrations of dendrimers in the values of positive charges per μmol/l in solution (Figs. 3 and 4). Flexible dendrimers (PAMAM g3, PAMAM g4, CBD-OS) and rigid ones (but small in size CBD-CS) in a concentration range of 0–100 μmol/l of '+' charges resulted in a small decrease of α-helices from 55 to ~48-45%, accompanied by the increase of unstructured state from 36 to 41–43%, and β-sheets from 9 to ~11% (Fig. 3A,B). In contrast, large and rigid dendrimers caused significant change in thrombin's secondary structure. The rigid phosphorus dendrimers of 3rd and 4th generations (CPD g3 and g4) at 0–100 μmol/l of '+' charges decreased α-helix content from 55 to ~35%, accompanied by an increase of the unstructured state from 36 to 50%, with β-sheets going from 9 to ~15% (Fig. 3A and B). Furthermore, the large rigid core hybrid dendrimers, SMT1 and SMT2, over range of 0–100 μmol/l of '+' charges markedly affected thrombin's secondary structure. There was a sharp decrease in α-helix content from 55 to ~2%, accompanied by the increase of the unstructured state from 36 to 61–62%, with β-sheets going from 9 to ~32% (Fig. 3A and B).

3.4. Fluorescence quenching (protein conformation)

All the dendrimers added to solution quenched the fluorescence of thrombin, thereby reflecting its conformational changes (Fig. 4). The data on conformational changes of thrombin are in a good

Table 1
Constants of binding (K_b) and the number of binding centers (n) per molecule of thrombin (n) of different dendrimers, obtained by circular dichroism (CD) and fluorescence quenching (F).

Dendrimer	K_b^{CD}, l/mol	n^{CD}	K_b^{F}, l/mol	n^{F}
PAMAM g3	$1.8 \pm 0.2 \times 10^5$	2.4 ± 0.2	$4.4 \pm 0.1 \times 10^5$	1.3 ± 0.2
PAMAM g4	$3.0 \pm 0.3 \times 10^5$	2.6 ± 0.3	$5.3 \pm 0.2 \times 10^5$	1.0 ± 0.2
NN16	$1.0 \pm 0.2 \times 10^5$	3.3 ± 0.3	$3.0 \pm 0.2 \times 10^5$	2.2 ± 0.4
BDBR 11	$2.9 \pm 0.3 \times 10^5$	2.6 ± 0.2	$3.7 \pm 0.2 \times 10^5$	1.7 ± 0.3
CPD g3	$4.5 \pm 0.4 \times 10^5$	1.3 ± 0.3	$11.0 \pm 0.1 \times 10^5$	1.0 ± 0.2
CPD g4	$5.9 \pm 0.3 \times 10^5$	1.1 ± 0.4	$12.0 \pm 0.2 \times 10^5$	1.2 ± 0.3
SMT1	$7.9 \pm 0.2 \times 10^5$	0.9 ± 0.2	$8.0 \pm 0.2 \times 10^5$	1.0 ± 0.2
SMT2	$11.2 \pm 0.4 \times 10^5$	0.5 ± 0.1	$10.3 \pm 0.1 \times 10^5$	0.7 ± 0.2

agreement with data on changes in its secondary structure. Over the range of 0–100 μmol/l of '+' charges, the changes in thrombin's secondary structure reflected its conformational changes for all the NPs tested. The effect of dendrimers depended on their surface charge, nature and generation – the highest generations of more rigid structure had the biggest effect. The strongest effect was seen with CPD dendrimers and hybrid SMT dendrimers, whereas the others had a 'lag-phase': small changes occurred at low concentrations, with the significant changes being seen at the high concentrations. Most interesting was the similarity of the effects of CPD and SMT dendrimers, which can be explained by differences between fluorescence quenching and circular dichroism techniques. The former gives data that depend on accessibility of the Trp chromophore of thrombin to a quencher. In the case of a quencher, this accessibility is determined by positively charged dendrons attached to the SMT surface, making them similar in behavior to rigid phosphorus dendrimers.

4. Discussion

Based on microscopic, CD and fluorescence quenching data, we can conclude that 2 important processes take place: (i) the larger dendrimers bearing more positively charged groups affected thrombin in a more significant manner than small ones; (ii) the more rigid dendrimers markedly changed thrombin structure in contrast to more flexible dendrimers. In this regard, flexible dendrimers changed protein structure mainly from α-helix to a disordered state, whereas the rigid ones changed more from α-helix to β-sheets. It is noteworthy that protein structural changes from α-helices to β-sheets is like an amyloid fibrillation process [6].

Based on Eq. (1) [15], we estimated the binding constant (K_b) and the number of dendrimers bound per molecule of thrombin.

$$\log[(I_0\text{-}I)/I] = \log K_b + n\log[Q], \quad (1)$$

I_0 is intensity of fluorescence in the absence of quenching molecules, I the intensity of fluorescence in the presence of a quencher, [Q] the quencher's concentration, K_b the binding constant, and n the number of binding sites of thrombin molecule. For CD experiments I_0 was equaled to α-helices content (see Fig. 3A), for fluorescence – to F (Fig. 4).

The results are shown in Fig. 5 and Table 1. It follows from Fig. 5 and Table 1 that the constants of binding are differing for small and big dendrimers. The more rigid and larger the dendrimers, the higher are the constants of binding. As follows from Fig. 3, this effect can be explained by both the electrostatic interactions between the surface groups of dendrimers and thrombin and the higher degree of hydrophobic interactions due to the presence of hydrophobic cavities in big generations of dendrimers [3,5]. The K_b of PAMAM g4 to thrombin obtained by CD was practically equal to the K_b of PAMAM g4 to human serum albumin obtained from thermodynamic analysis [16]. As expected, the number of dendrimers bound by a single molecule of thrombin decreased with the increasing

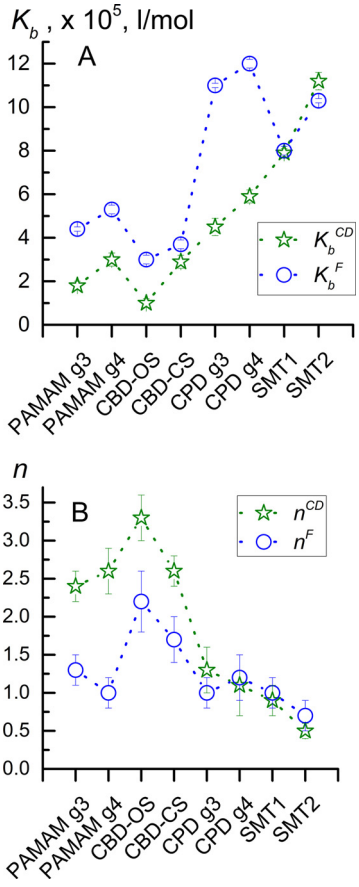

Fig. 5. Binding constants (K_b) and the number of binding centers (n) per molecule of thrombin (n) of different dendrimers obtained by circular dichroism (CD) and fluorescence quenching (F).

size of dendrimers. Interestingly K_b for dendrimer binding shown by CD was lower and n was higher than the ones from fluorescence quenching. This apparent contradiction might be explained by differences in these techniques. CD is based on absorption of all amino acid residues, whereas fluorescence quenching is based on quenching of the Trp residues in thrombin [17,18]. Thus changes in the% of α-helices after addition of dendrimers reflect the global changes in thrombin's secondary structure, whereas Trp fluorescence quenching reflects the local changes in the vicinity of Trp [17,18]. The large hybrid dendrimers, SMT1 and SMT2, induced quite radical changes in thrombin structure that their binding constants and n were equal for CD and fluorescence quenching measurements. A similar process, i.e. fibrillation of human serum albumin after addition of 6.75 μmol/l PAMAM-OH g5 dendrimer, had been reported by Sekar et al. [18]. In contrast, Nowacka et al. [19] showed a stabilizing effect of small concentrations (0.001–0.1 μmol/l) of PAMAM g3 and g4 dendrimers on insulin, whereas Heegaard et al. [20] reported destabilization of amiloid aggregates of prion peptide PrP106-126 under the action of dendrimers. Milowska et al. [21] showed an inhibiting effect of phosphorus dendrimers on the fibrillation of alpha-synuclein at low protein/dendrimer ratios (1:0.1 and 1:0.5) alone; a higher ratio did not show the same. Milowska et al. [22] also reported that PAMAM g4 dendrimer (0–2 μmol/l) inhibited fibrillation of alpha-synuclein, but PAMAM g3.5 dendrimers did not [22]. Thus the dual effect of dendrimers is seen: at low concentrations they can stabilize the structure of proteins, whereas at high concentrations they induce rearrangements of protein structure, leading to fibrillation and aggregation of proteins. Our results support the hypothesis of Tomalia et al. [23,24], who suggested dividing nanoparticles into 2 categories, the 'hard' and the 'soft' [23,24].

'Hard' nanoparticles include metal nanoparticles, fullerenes, carbon nanotubes and quantum dots. 'Soft' nanoparticles include liposomes, dendrimers, polymer micelles and polymeric nanocapsules. These 2 classes will clearly have different interactions with proteins based on significant differences in their surface density that determine their hydrophobicity and surface charge density [23,24].

Thus, the intensity of interactions between dendrimer and thrombin depends strongly on (a) the nature of the dendrimer, and on (b) its size, (c) surface charge, (d) flexibility/rigidity. Two important processes have been observed: i) the larger dendrimers with more positively charged groups had a greater effect on than the small ones; ii) the more rigid dendrimers markedly changed thrombin secondary structure in contrast to the more flexible dendrimers. Dendrimers changed the protein structure mainly from α-helix to a disordered state whereas SMTs changed it from α-helices to β-sheets. These mechanisms are important to our understanding of protein-nanoparticle interactions.

Acknowledgements

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