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The Interaction between Polycationic Poly-Lysine Dendrimers and Charged and Neutral Fluorescent Probes

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Abstract The interactions between polycationic poly-lysine dendrimers and hydrophobic fluorescent probes (anionic ANS and neutral Prodan) were studied. R121 and R131 dendrimers were not able to interact with anionic and neutral hydrophobic groups. R124 was able to interact with neutral and anionic hydrophobic fluorescent probes, however mainly through hydrophobic forces. Dendrimers R155 and R169 showed the maximal effects. The strongest interactions observed for R169 can be explained by intramolecular folding (stacking) of its two L-proline residues. Using double fluorescence titration technique for ANS probe allowed to receive such constant of binding and the number of binding centers: for R121, $1.8 \cdot 10^3$ $(mol/l)^{-1}$ and 1.07; for R124, $12.1 \cdot 10^3$ $(mol/l)^{-1}$ and 0.48; for R131, $4.7 \cdot 10^3$ $(mol/l)^{-1}$ and 0.48; for R155, $9.2 \cdot 10^3$ $(mol/l)^{-1}$ and 1.36; for R169, $39.6 \cdot 10^3$ $(mol/l)^{-1}$ and 0.97. Thus, neutral and anionic hydrophobic probes can be used for the fast preliminary screening of binding properties of newly synthesized polycationic dendrimers.

Keywords Fluorescent probe · ANS · Prodan · Cationic poly-lysine dendrimers · Electrostatic and hydrophobic interactions · Binding constant

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Introduction

Dendrimers are a relatively novel class of polymers. Since they were discovered in the early 1980's by Tomalia, Newkome and Vögtle groups [1–3], the use of dendrimers as carrier systems, anticancer, antimicrobial and antifungal compounds is gaining popularity. Dendrimers having surface peptides grafted onto a traditional dendrimer framework and these incorporating amino acids into the framework as branching or core units are both defined as "peptide dendrimers." Among amino acid branching units from which dendrimers can be assembled lysine is the most common [4]. Polylysine peptide dendrimers, because of their better solubility in water, better stability to proteolysis and smaller toxicity to the human cells than their linear polymeric analogs [5] can be used in many biomedical applications. They were found to work as non–viral gene delivery carriers with low cytotoxicity [6].

On the other hand, the modification of the structure of poly-lysine dendrimers can drastically increase their antimicrobial, antifungal and antiviral properties [7–9]. Specially synthesized polycationic poly-lysine dendrimers were proposed as antimicrobial agents [9, 11–13]. For example, lysine dendrimers have been used as synthetic scaffolds for attachment of two to eight copies of a tetrapeptide R4 (RLYR) or an octapeptide R8 (RLYRKVYG). Both R4 and R8 have been found in protegrins and tachyplesins—natural peptides with antimicrobial activity. High potency in antimicrobial assays against 10 organisms in high- and low-salt conditions was found in this group of compounds [13]. Another structural approach in this area was construction of low molecular mass lysine-based peptide dendrimers designed as branched analogues of the natural cationic antimicrobial peptides [12] (Fig. 1). They represent a new class of membrane-active dendrimers, where dendrimer

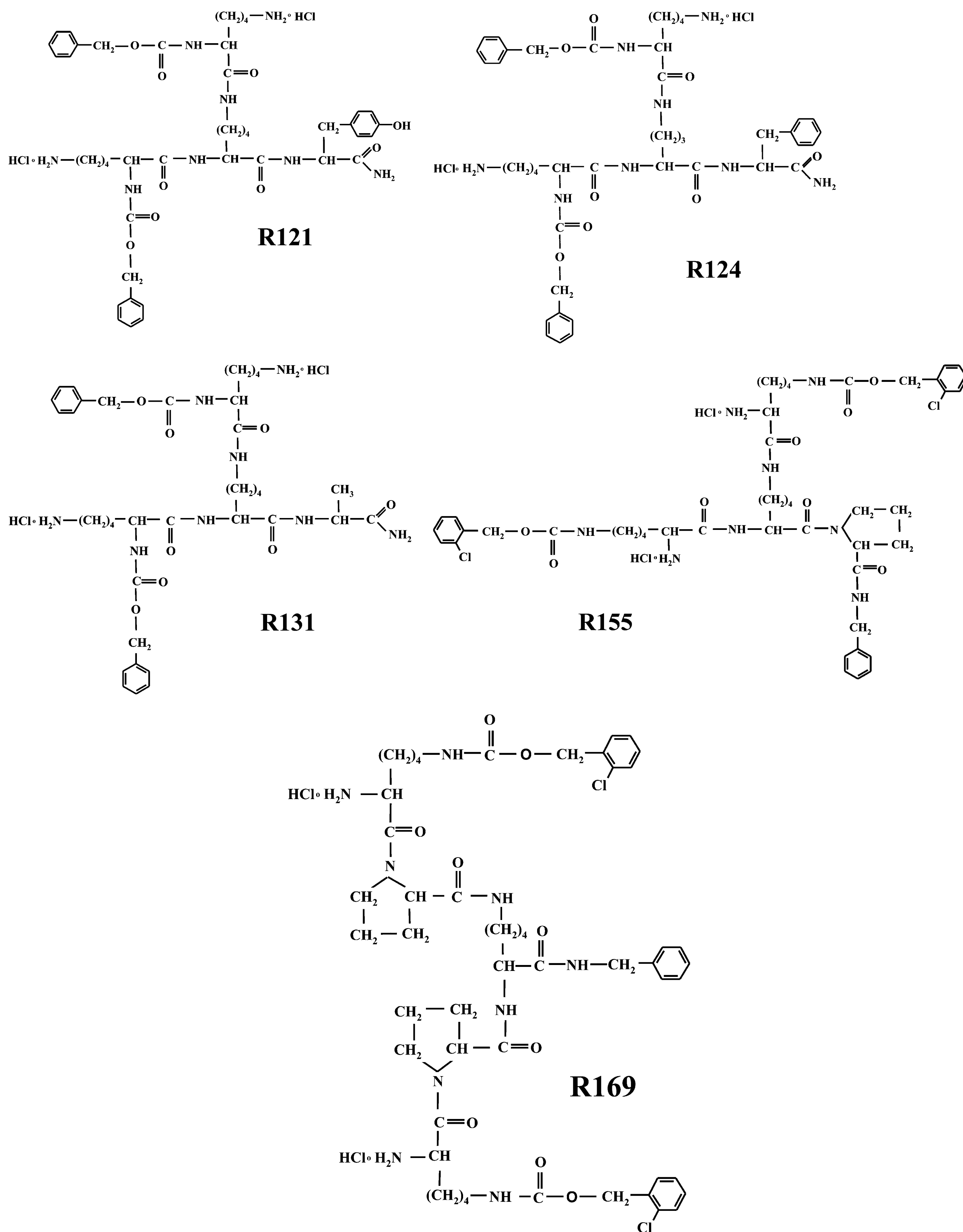

Fig. 1 The chemical structures of polylysine dendrimers. For more details see Introduction

tree is used not only for multiplication of active elements but also for spatial distribution of cationic and aromatic (hydrophobic) groups, that is essential for interactions with bacterial membranes. These dendrimeric peptides expressed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. Although they are structurally different that any other of the above types of compounds, it appears that both linear endogenous antimicrobial peptides and their synthetic branched cationic analogs have ability to disrupt bacterial membranes. This structural dissimilarity raises several questions about mechanism of their interactions with target membranes and other elements constituting living cells [9, 12]. It was proposed [9] that steric distribution and type of hydrophobic (aromatic) groups and cationic centers are important components of dendrimeric structure and influence both antimicrobial potency and toxicity. This paper presents results on hydrophobic and electrostatic interactions between poly-lysine dendrimers and fluorescent probes.

The ANS (8-aniline-1-naphtalene sulfonate) is a fluorescent probe for the detection and analysis of folding and unfolding processes in proteins, their binding properties and in the studies of biological membranes [15]. After binding, ANS fluorescence depends on how the ground and excited state of the molecule interfaces with the surrounding environment. If water molecules in contact are immobilized and cannot quench, or if they are mobile and can do it, these are the factors influencing molecule fluorescence. The parameters of ANS binding to proteins and membranes are estimated using double fluorimetric titration technique [15–17]. This technique was applied to estimate binding properties of dendrimers [18, 19].

Prodan is a neutral fluorescent probe. It also has extensive solvent polarity–dependent fluorescence shifts (from 401 nm in cyclohexane to 531 nm in water) [20–22]. It is sensitive to polar environment in lipid bilayers, micelles and even in the membrane [21–24]. In the gel-state of phospholipids bilayer it has the maximum of fluorescence emission at 437 nm [23].

The above mentioned properties of fluorescent probes ANS and Prodan were used to estimate the impact of hydrophobic and electrostatic interactions on the interaction between poly-lysine dendrimers and biomolecules.

Materials and methods

Dendrimer synthesis

All tested dendrimers were synthesized in solution step-by-step procedure using commercial Boc-Z-or 2-Cl-Z-protected amino acids [25]. For coupling reactions, N,N-dicyclohexylcarbodiimide in presence of hydroxybenzotriazol (HOBt) and di-phenylethylamine (DIPEA) was used. Hydrochloric acid in acetic acid has been used for Boc-group deprotection. In the last step of synthesis peptides were converted into an amide form by treating methyl esters by ammonia overnight. Crude peptide dendrimers have been purified using gel filtration on Sephadex LH-20 in methanol, followed by preparative HPLC in water/ethyl acetate system. All dendrimers were confirmed to have corrected amino acid analysis molecular weights and purity by ESI-MS technique analyzing both molecular peak and fragmentation ions [9, 12].

Materials

1-anilinonaphtalene-8-sulphonic acid (ANS) was purchased from Sigma-Aldrich (USA). 6-propionyl-2-dimethylamino-naphthaline (Prodan) from Molecular Probes (Invitrogen). The other chemicals were of analytical grade. Dendrimer solutions were prepared using 18.2 MΩ Milli-Q water (Millipore), while ANS and Prodan were dissolved in dimethylsulfoxide (DMSO).

Fluorescence of ANS

Fluorescence spectra were taken with a Perkin-Elmer LS-50B spectrofluorimeter at room temperature (23°C). The excitation and emission wavelengths were set at 370 nm and between 400 and 600 nm respectively. Excitation and emission slit widths were 5 and 2.5 nm, respectively. During measurements samples were continuously stirred in 1-cm path length quartz cuvettes.

The binding constant (K_b) and the number of binding sites per one dendrimer molecule (n) were estimated using double fluorimetric titration method [16–19]. In the first ANS titration, dendrimers' concentration (D) was increased, while ANS concentration remained constant. Theoretical approximation was made to obtain maximum fluorescence intensity of the ANS fluorescent probe (F_{max}). When the fluorescence intensity growth was no longer observed it was concluded that all ANS molecules that had possibility to bind were bound to dendrimers. To obtain the specific fluorescence intensity (F_{sp}) for the bound fluorescent probe, maximum fluorescence was divided by probe concentration used for the first titration (C^1_{ANS}):

$$F_{sp} = \frac{F_{max}}{C^1_{ANS}}, \quad (1)$$

Contrary to the first fluorimetric titration in the second titration dendrimers had constant concentration (C_D), while ANS concentration was increased (C^2_{ANS}). Then, fluorescence intensity (F) was measured. The concentration of ANS probe bound by dendrimers was calculated using equation:

$$C^{bound}_{ANS} = \frac{F}{F_{sp}}, \quad (2)$$

Concentration of free ANS molecules was determined as follows:

$$C_{\mathrm{ANS}}^{\mathrm{free}} = C_{\mathrm{ANS}}^{2} - C_{\mathrm{ANS}}^{\mathrm{bound}}. \tag{3}$$

Binding constants for the fluorescent probe molecules (K_b) and the number of ANS binding centers in solution (N) can be can be calculated from the formula:

$$\frac{1}{C_{\mathrm{ANS}}^{\mathrm{bound}}} = \frac{1}{K_b \cdot N \cdot C_{\mathrm{ANS}}^{\mathrm{free}}} + \frac{1}{N}, \tag{4}$$

and afterwards presented as the graph of the function of $1/C_{\mathrm{ANS}}^{\mathrm{bound}}$ versus $1/C_{\mathrm{ANS}}^{\mathrm{free}}$ (Fig. 4). The curve initial region is a straight line. After modifying the formula (4) by replacement of the number of binding centers in solution (N) with the number of binding centers per one dendrimer molecule (n), finally the equation was:

$$\frac{C_D}{C_{\mathrm{ANS}}^{\mathrm{bound}}} = \frac{1}{K_{\mathrm{b}} \cdot n \cdot C_{\mathrm{ANS}}^{\mathrm{free}}} + \frac{1}{n}, \tag{5}$$

where:

$$n = \frac{N}{C_D} \tag{6}$$

For calculations the corrected fluorescence values were used, as follows. (1) For the first titration (concentration of ANS constant), $F^1 = F^{\mathrm{N}} - F_{\mathrm{ANS}}^{0} - F_{\mathrm{Dendrimer}}^{\mathrm{N}}$, where fluorescence F^1 at a concentration point N is the difference between the fluorescence of the dendrimer + ANS system (F^{N}), that of pure ANS at a concentration point 0 (F_{ANS}^{0}), and that of pure dendrimer at a concentration point N ($F_{\mathrm{Dendrimer}}^{\mathrm{N}}$). In our conditions F_{ANS}^{0} was 16.5 ± 1.2. (2) For the second titration (concentration of dendrimer constant), $F^2 = F^{\mathrm{N}} - F_{\mathrm{ANS}}^{\mathrm{N}} - F_{\mathrm{Dendrimer}}^{\mathrm{N}}$, where the fluorescence value F^2 was equal to the difference between the fluorescence the dendrimer + ANS system (F^{N}), that of pure ANS (fluorescence of ANS at a concentration point N, i.e. $F_{\mathrm{ANS}}^{\mathrm{N}}$), and that of pure dendrimer at a concentration point 0 ($F_{\mathrm{Dendrimer}}^{0}$). According to these equations, all "control" points in Figs. 1 and 2 (points without ANS for the first titration and points without dendrimer for the second titration) are equal to zero (no effect).

Fluorescence of Prodan

Fluorescence spectra were taken with a Perkin-Elmer LS-50B spectrofluorimeter at room temperature (23°C). The excitation wavelength for Prodan was set at 340 nm and the emission range was set between 400 and 600 nm. The excitation and emission slit widths were 10 nm and 5 nm,

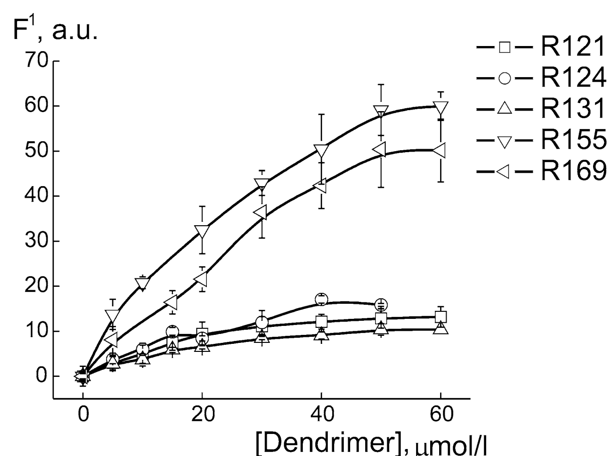

Fig. 2 Changes in intensity (F^1) of ANS fluorescence maximum upon addition of dendrimers in different concentrations. Concentration of ANS was 5 μmol/l. $\lambda_{\mathrm{ex}} = 370$ nm. $\lambda_{\mathrm{em}} = 500$ nm. For definition of F^1 see Materials and Methods

respectively. Fluorescence F (presented at Fig. 4) at a concentration point N is the difference between the fluorescence of the 'dendrimer + Prodan' system (F^{N}), that of pure Prodan at a concentration point 0 (F_{Prodan}^{0}), and that of pure dendrimer at a concentration point N ($F_{\mathrm{Dendrimer}}^{\mathrm{N}}$), i.e. $F = F^{\mathrm{N}} - F_{\mathrm{Prodan}}^{0} - F_{\mathrm{Dendrimer}}^{\mathrm{N}}$. In our conditions F_{Prodan}^{0}n was 21.4 ± 1.1. Samples were contained in 1 cm path length quartz cuvettes and were continuously stirred.

Light scattering

Light scattering was estimated using Perkin-Elmer LS-50B spectrofluorimeter at room temperature (20°C). The excitation and emission wavelengths were set at 650 nm. The excitation and emission slit widths were 2.5 nm. Samples were contained in 1 cm path length quartz cuvettes and continuously stirred.

All data are expressed as a mean value ± SD of 6 independent experiments. Statistical significance was assessed using Student–Fisher test.

Results and discussion

ANS binding

The dendrimers used have different structures. They can be divided into two groups: to the first group belong R121, R124 and R131 dendrimers, which have α-amino groups protected by benzyloxycarbonyl groups; the second group is created by two dendrimers R155 and R169, which possess ε-amino groups protected by 2-chloro benzyloxycarbonyl groups. Furthermore, only last two have hydrophobic L-proline residue in their structure. All previously made

experiments showed that these two dendrimers are the most toxic [9, 12]. As a result of dendrimers different chemical structures the differences between their ANS probe binding properties can be noticed.

Some authors claim that ANS binds to cationic groups of proteins and that its binding depends on ion-pair formation of the molecule [14] but it is also said that ANS binds preferentially to hydrophobic cavities of the proteins [26]. When ANS probe is bound to hydrophobic sites of membrane or protein it fluoresces strongly. As it can be seen from Fig. 2 dendrimers addition is followed by the ANS fluorescence increase [27]. Similar effect is noted when ANS concentration is increased and dendrimers concentration remains constant (Fig. 3). The comparison of n and K_b of dendrimers shows that the biggest association constants characterize R169, R124 and R155 dendrimers. Due to the fact that all dendrimers used possess similar number of cationic groups, the differences between dendrimers affinity to the fluorescent probe must be the result of other factors than polar interactions. Because of aromatic structure of ANS, R124, R155 and R169 dendrimeric macromolecules may bind fluorescent probe through aromatic–aromatic interactions [26]. It can be suggested that prolines may play an important role in the dendrimer–probe interactions, because their aliphatic nature is sufficient to bind hydrophobic moieties of ANS [28]. Following the data R169 dendrimer has the strongest affinity to the ANS probe. This can be the result of the presence of two proline residues in the molecule. R155 dendrimer, which also possess this amino-acid residue has also a strong affinity to ANS, although it is weaker than in the case of R169. However, it is possible that R169 structure is folding and the hydrophobic L-proline groups are stacking with each other and/or with benzene groups [29–32]. Because of this the hydrophobic interactions become weaker and the affinity towards ANS is more due to electrostatic forces and becomes

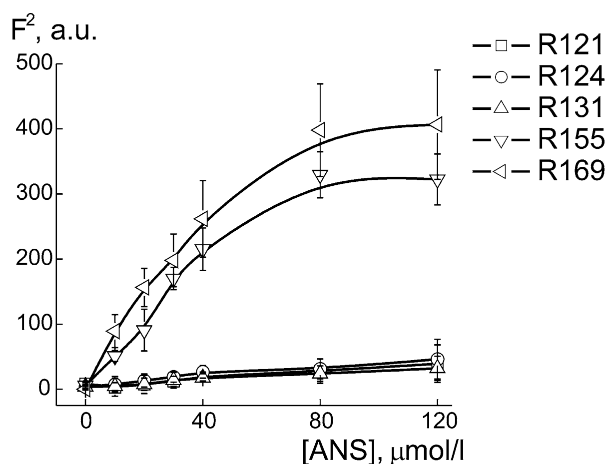

Fig. 3 The dependence of intensity (F^2) of ANS fluorescence maximum on ANS concentration in the presence of 30 μmol/l dendrimers. $\lambda_{ex} = 340$ nm. $\lambda_{em} = 500$ nm. For definition of F^2 see Materials and Methods

Table 1 Binding constants (K_b) and the number of binding centers per one dendrimer molecule (n) for ANS binding by poly-lysine dendrimers

	R121	R124	R131	R155	R169
n	1.07	0.48	0.48	1.36	0.97
K_b [M^{-1}]	1.8×10^3	12.1×10^3	4.7×10^3	9.2×10^3	39.6×10^3

stronger. High affinity constant noted for R124 dendrimer, although its structure is similar to R121 dendrimer, can be affected by the replacement of hydrophilic tyrosine by hydrophobic amino acid – phenylalanine.

The number of binding sites for ANS (Table 1) is different for different types of small–mass polylysine molecules. From Stachard-Klotz plot we got that for both R124 and R131 n was 0.5 (Fig. 4). Thus, seemly at least two dendrimer molecules are needed for effective enhancement of ANS fluorescence. Probably, one dendrimer provides hydrophobic interaction with ANS, whereas the second is necessary for a hydrophobic shield for ANS bound to the first one. The other dendrimers interact with fluorescent probe with 1:1 stoichiometry.

Interaction with Prodan and light scattering

In our conditions the pure Prodan in water had the fluorescence emission maximum at 520 nm for the excitation at 340 nm. Addition of dendrimers led to appearance of Prodan fluorescence maximum at 430 nm. Figure 5 presents the dependence of Prodan fluorescence at 430 nm on dendrimer concentration. As follows from Fig. 5 the maximal effect was observed for two dendrimers: R124 and R155.

The light scattering by dendrimers is presented in Fig. 6. As follows from Figs. 5 and 6, the increase of Prodan fluorescence corresponds to aggregation of dendrimers (when Prodan incorporates into dendrimer aggregates). Two dendrimers showed significant aggregation. R124 is slightly

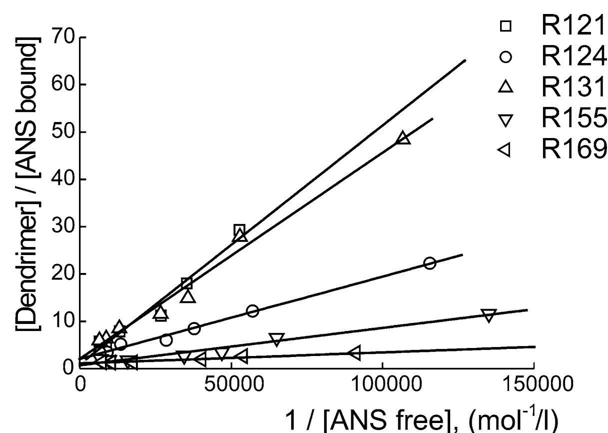

Fig. 4 Scatchard-Klotz plots for ANS binding by poly-lysine dendrimers. For details see Materials and Methods

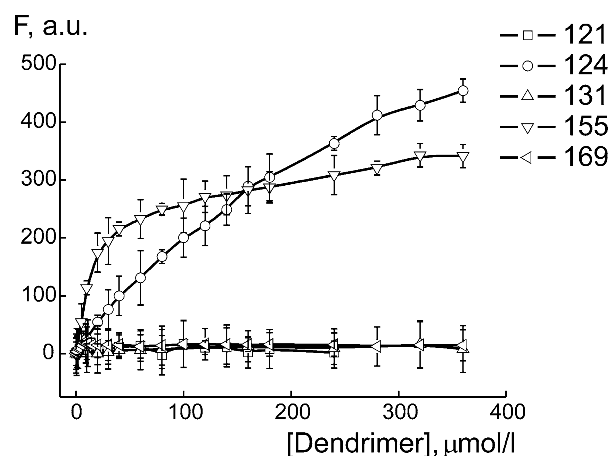

Fig. 5 The dependence of Prodan fluorescence emission at 430 nm on dendrimer concentration. Concentration of Prodan was 2.5 μmol/l. $\lambda_{ex} = 340$ nm, $\lambda_{em} = 430$ nm. For definition of F see Materials and Methods

soluble in water and self-aggregates after injection, while R155 forms the stable large aggregates of constant diameter. Unlike these two dendrimers, other dendrimers form monomers or very small aggregates (micelles). The possibility of precipitation was excluded by investigating changes in the absorption spectra. Absorption spectra of the pure Prodan probe possessed maximum at $\lambda_{max} = 354$ nm. Addition of dendrimers, even 50 μM, caused maximum shift to $\lambda_{max} = 359$ nm, what means that dendrimers aggregate rather than precipitate. Due to the fact of self–aggregation of Prodan probe in our conditions in the concentrations above 8–10 μM it was not possible to use double fluorimetric titration method.

The results show that hydrophobic interactions play the main role in interactions between Prodan and dendrimers. R169 does not practically interact with neutral Prodan and does not form the large micelles/aggregates. But it interacts

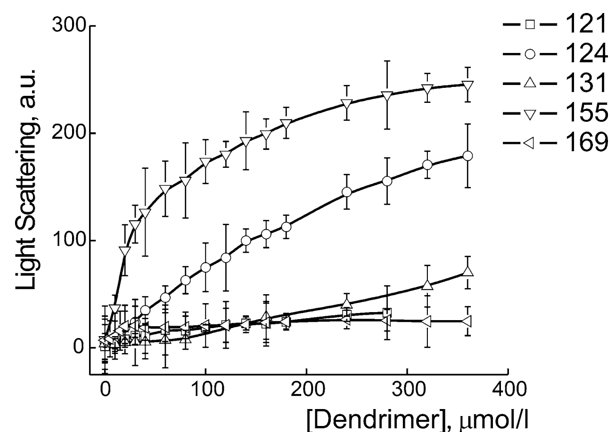

Fig. 6 The dependence of light scattering by dendrimers on their concentration. $\lambda_{ex} = 650$ nm, $\lambda_{em} = 650$ nm. For details see Materials and Methods

with high efficiency with anionic ANS. Also, it is easy to notice that the concentrations of dendrimers used in studies on ANS-dendrimer interactions are not big enough to cause aggregation (do not directly correspond with processes of aggregation and are much less).

The comparison of R121, R124 and R131 shows that R121 and R131 are soluble in water but inert. They are not able to interact with charged or hydrophobic groups of fluorescent probes or self-associate, whereas R124 which is much more hydrophobic (replacement of hydrophilic tyrosine by hydrophobic phenylalanine) and is able to interact (first of all, hydrophobically) with ANS and Prodan.

The comparison of R155 and R169 shows that R169 (two hydrophobic L-proline groups) reveals pronounced electrostatic interactions and slight hydrophobic interactions. The difference between R155 and R169 shows the intramolecular folding of L-proline residues through the hydrogen bonding of their helical structures [29–32]. During this process the hydrophobicity of R169 significantly decreases [31]. This folding can explain both the slight interactions of R169 with Prodan and the strong interaction with charged ANS.

Conclusions

The difference in antimicrobial properties of polycationic polylysine dendrimers and their toxicity can be explained by differences in their hydrophobicity and charge. R121 and R131 are not able to interact with anionic and neutral hydrophobic groups that well correlates with their low antimicrobial properties [9]. In contrast, the third member of this group—R124 (having quite high antimicrobial properties and toxicity [9]) is able to interact hydrophobically with neutral and anionic hydrophobic fluorescent probes. Dendrimers R155 and R169 show the maximal effects. R155 interacts preferentially with neutral Prodan by hydrophobic interactions, while R169 interacts not only through hydrophobic, but also electrostatic ones. Weaker hydrophobic interactions in R169 can be explained by intramolecular folding (stacking) of its two L-proline residues. Thus, ANS probe is a useful tool for studies on binding and electrostatic interactions of polycationic dendrimers while Prodan can be used for studies on dendrimers aggregation and estimation of their hydrophobic properties.

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