Binding Properties of Polyamidoamine Dendrimers

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ABSTRACT: Dendrimers are globular, hyperbranched polymers possessing a high concentration of surface functional groups and internal cavities. These unique features make them good host molecules for small ligands. To reveal relationships between dendrimer size and its encapsulating properties, the interactions of the fourth and the sixth generations of polyamidoamine dendrimers (PAMAM G4 and PAMAM G6) with a fluorescent dye 1-anilinonaphthalene-8-sulfonate (ANS) were studied. Because ANS is a fluorescent molecule and its fluorescence is very sensitive to changes in its microenvironment, it was possible to use spectrofluorometric methods to evaluate the interactions with dendrimers. A double fluorometric titration method was used to estimate a binding constant and the number of binding centers. There were two types of dendrimer binding centers characterized by different affinity towards ANS. For PAMAM G4, the values of K_b and n for low-affinity and high-affinity sites equaled to 2.6×10^5, 0.60 and 3.70×10^6, 0.34, respectively, whereas in the case of PAMAM G6, these values equaled to 1.2×10^5, 76.34 and 1.38×10^6, 22.73. It was observed that the size of the dendrimer had a strong impact on the number of ANS molecules that interacted with dendrimers and their location within the macromolecule. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 2036–2040, 2007

Key words: dendrimers; fluorescence; host-guest systems; ANS; PAMAM

INTRODUCTION

Dendrimers are a relatively new class of polymers with a well-defined molecular structure that has a big impact on their applications. They are synthesized from a polyfunctional core by adding branched monomers that react with the functional groups of the core molecule, in turn, leaving end groups that can react again.[1] The number of reactive terminal groups increases after the addition of the layer of monomers. The more layer of branched units are attached, the higher the number of the generation of the dendrimer. Therefore, dendrimers are globular, hyperbranched macromolecules, which possess a high concentration of surface groups. The presence of empty, internal cavities is the next consequence of their specific synthesis. These unique properties make dendrimers suitable for drug delivery systems.[2,3] Drug molecules can either be attached to the end groups of the dendrimer or encapsulated in the macromolecule interior. Because of the large number of terminal groups, one dendrimer molecule is capable of carrying drugs at a high density.[4] On the other hand, drugs encapsulated inside the dendrimer can be protected from degradation or released slowly, which is an important factor in the reduction of therapeutic agents toxicity and in the avoidance of side effects.[5] Both strategies of applications are very promising in targeted antitumor therapy.

The ability to encapsulate small molecules inside dendrimer strongly depends on the size of the dendrimer. Lower generations (from first to third) are believed to be too small to effectively interact with guest molecules, because their shape is rather flat and ellipsoidal than globular. On the other hand, dendrimers of very high generations can create a dense shell on the surface that enables the incorporation of ligands.[6] To reveal more details on the relationship between the dendrimer structure and its properties, we decided to examine two generations of polyamidoamine dendrimers: fourth and sixth. Additionally, we wanted to investigate how deep anilinonaphthalene-8-sulfonate (ANS) can locate within dendrimers.

Polyamidoamine (PAMAM) dendrimers are based on an ethylenediamine core and branched units are built from methyl acrylate and ethylenediamine. The fourth generation of PAMAM dendrimers (PAMAM G4) possesses 64 amino groups on a surface whereas PAMAM G6 dendrimers have 256 amino groups at chain-ends. Molecular weight for PAMAM G4 and PAMAM G6 equals to 14,215 and 58,048 Da, and a diameter 4 and 6.7 nm, respectively.[7]

1-Anilinonaphtalene-8-sulfonic acid (ANS) was a model of a guest molecule in our studies. This is a very convenient model since ANS is a fluorescent molecule and its fluorescence is sensitive to the changes in its

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microenvironment.[8] Therefore, it was possible to evaluate its interactions with dendrimers by spectrofluorometric methods.

MATERIALS AND METHODS

Dendrimers (PAMAM G4 and PAMAM G6) were purchased from Dendritic NanoTechnologies (USA). 1-Anilinonaphthalene-8-sulfonic acid was obtained from Sigma (USA). All the other chemicals were of analytical grade. Double-distilled water was used to prepare the solutions. Anilinonaphthalene-8-sulfonate (ANS) was dissolved in phosphate-buffered saline (PBS: 150 m*M* NaCl, 1.9 m*M* NaH_2PO_4, 8.1 m*M* Na_2HPO_4, pH 7.4) at concentrations of 100 μM.

Fluorescence emission spectra and synchronous spectra were taken with a PerkinElmer LS-50B spectrofluorometer at room temperature. In the case of the emission spectra, the excitation wavelength was set at 360 nm and the emission spectra were registered between 400 and 700 nm. It was checked that dendrimers were not excited by 360 nm wavelength and did not emit fluorescence. When emission spectra are not sufficient to distinguish between two or more peaks, synchronous spectra can provide useful information. Synchronous scanning is based on simultaneous scanning at a constant shift between the excitation and emission monochromators. For synchronous scan spectra, the initial (excitation) wavelength was set at 300 nm and the wavelength shift $\Delta\lambda$ was equal to 122 nm. The excitation and emission slit widths were 7 and 4 nm, respectively. Samples were contained in 1 cm path length quartz cuvettes and were continuously stirred.

The binding constant (K_b) and the number of binding centers per one dendrimer molecule (n) were determined by a double fluorometric titration technique and calculated using Scatchard-Klotz analysis.[9] In the first fluorometric titration, the dendrimer at increasing concentrations was added to the solution of ANS at constant concentration and the maximum intensity (F_{max}) of ANS fluorescence was recorded. It corresponded to the state when all ANS molecules were bounded by the dendrimer. The maximum fluorescence intensity of ANS divided by its concentration gave the specific fluorescence intensity for the bound probe (F_{sp}):

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

where C^1_{ANS} is the ANS concentration during the first fluorometric titration.

In the second fluorometric titration, the system was reversed and ANS was added to the dendrimer solution and the fluorescence intensity (F) was measured. The concentration of ANS bound by the dendrimer was calculated as

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

and concentration of free ANS as

$$C^{free}_{ANS} = C_{ANS} - C^{bound}_{ANS} \quad (3)$$

The binding constant (K_b) and the number of binding centers per one molecule of the dendrimer (n) were determined from the plot of $C_{dendrimer}/C^{bound}_{ANS}$ on the ordinate versus $1/C^{free}_{ANS}$ on the abscissa, according to the equation

$$\frac{C_{dendrimer}}{C^{bound}_{ANS}} = \frac{1}{K_b\, n\, C^{free}_{ANS}} + \frac{1}{n} \quad (4)$$

RESULTS AND DISCUSSION

The fluorescence of ANS is sensitive to changes in the polarity around the chromophore, because it has higher dipole moments in the excited state than in the ground state. When there are interactions with solvent dipoles, some of the energy of the excited state is lost, and so the emission is red-shifted. The more polar the environment, the bigger the shift.[10] ANS has a low fluorescent yield in a polar environment, and it is

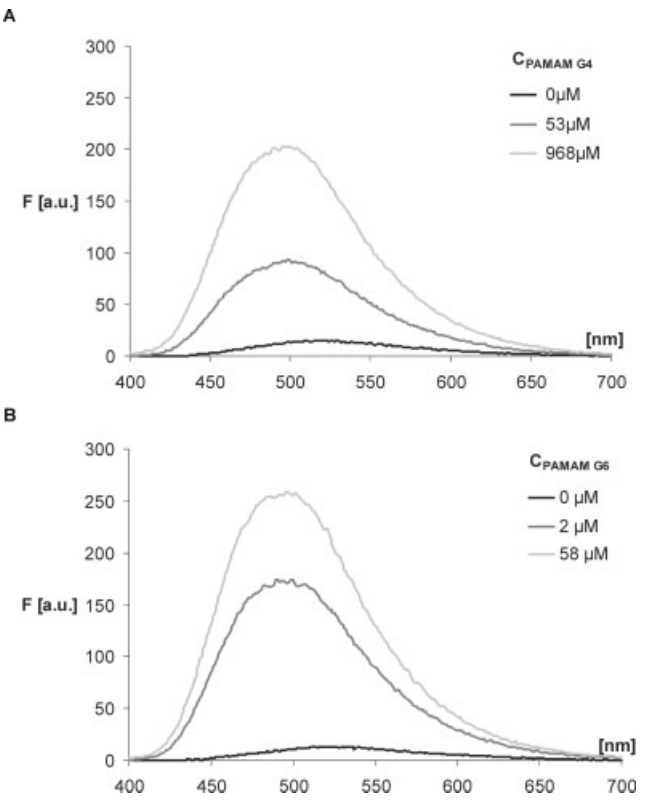

Figure 1 Fluorescence emission spectra of ANS (100 μM) in the presence of PAMAM G4 (A) and PAMAM G6 (B). λ_{exc} = 360 nm; scan speed 400 nm/min.

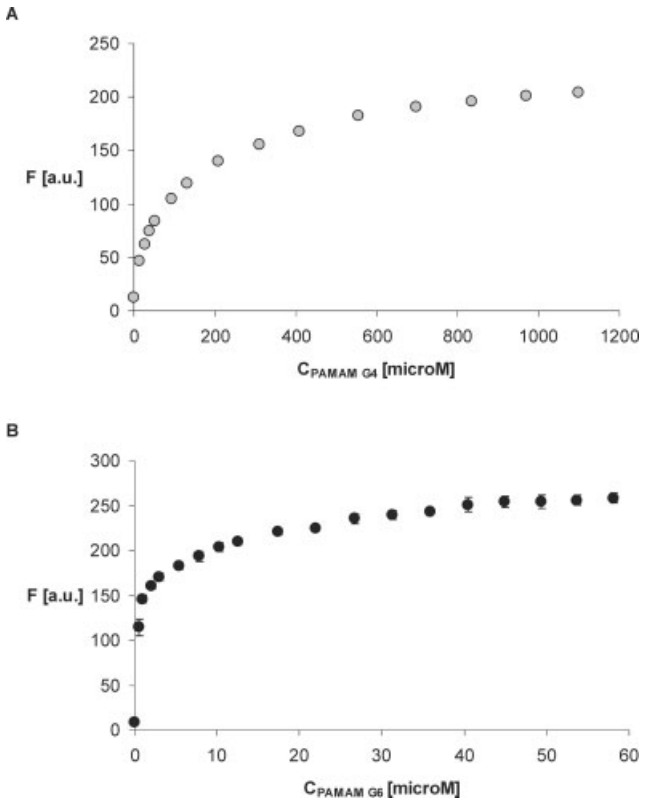

Figure 2 Fluorescence intensity of ANS for $\lambda = 495$ nm upon addition of PAMAM G4 (A) and PAMAM G6 (B). $\lambda_{exc} = 360$ nm. The values of standard errors (bars on the graphs) did not exceed 8%.

greatly enhanced as the solvent polarity decreases. It happens because ANS is very sensitive to water molecules that dynamically quench its fluorescence.[11]

The pure ANS probe in aqueous solution had a weak fluorescence in a range 400–600 nm with a maximum at 525 nm. The ANS fluorescence increased significantly upon addition of both PAMAM G4 and PAMAM G6 dendrimers (Fig. 1). These results are in a good agreement with our previous results.[12,13] Adding dendrimers led to a blue shift of the emission maximum (λ_{max}) to about 495 nm. It indicates that ANS was placed in a more hydrophobic environment due to its interactions with dendrimers. Nevertheless, when we compare the value of λ_{max} in the presence of dendrimers with the values that were obtained for ANS bound to human serum albumin that equaled to 465 nm, we can conclude that ANS does not locate in dendrimers as deep as in the protein.[14]

Recording the fluorescence intensity for λ_{max} gave hyperbolic curves (Fig. 2), from which values of F_{sp} were calculated. Similarly to the first titration, the titration of dendrimer solutions with ANS also resulted in the enhancement of fluorescence intensity accompanied by a blue shift. Changes in the fluorescence intensity for λ_{max} upon the addition of aliquots of ANS were registered (Fig. 3). On the basis of these curves, the Scatchard's plots with $C_{dendrimer}/C_{ANS}^{bound}$ on the ordinate versus $1/C_{ANS}^{free}$ on the abscissa were drawn (Fig. 4). The hyperbolic shape of these plots revealed that there were two types of binding centers where ANS was located that were characterized by a different degree of affinity. The inflection point was estimated using a segment regression. A linear regression for the both parts of the graph gave the values of K_b

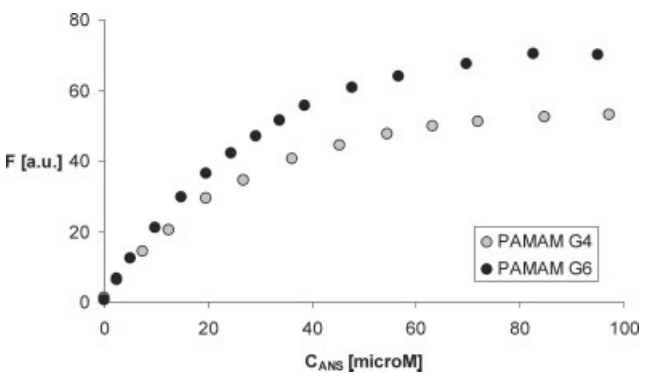

Figure 3 Fluorescence intensity of ANS for $\lambda = 495$ nm upon addition of ANS. $C_{PAMAM\ G4} = 60$ μM, $C_{PAMAM\ G6} = 5$ μM, and $\lambda_{exc} = 360$ nm. Standard errors did not exceed 10%.

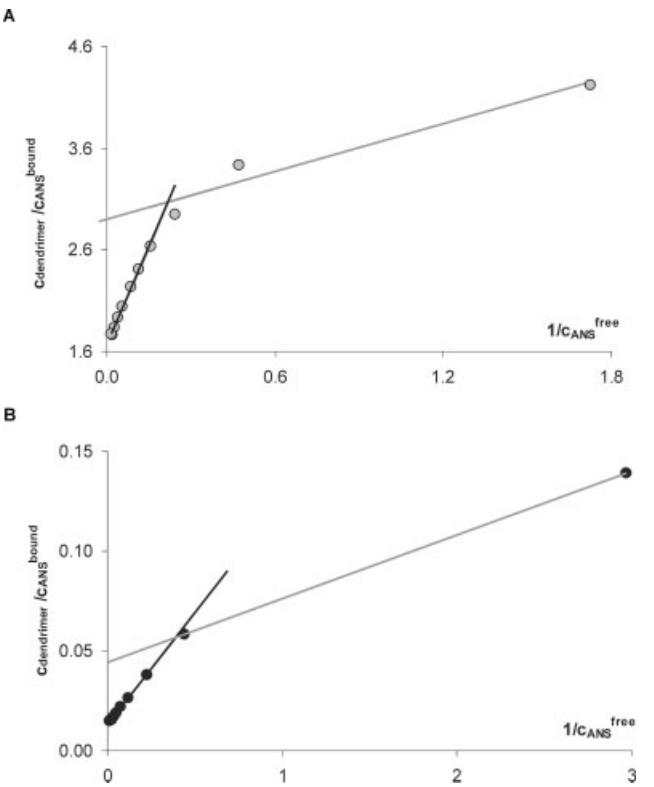

Figure 4 Scatchard's plots for binding ANS-PAMAM G4 (A) and ANS-PAMAM G6 (B). Each point is the average of six independent experiments. Standard errors did not exceed 10%.

TABLE I
The Values (±SE) of Binding Constants of ANS and Numbers of Binding Sites in PAMAM Dendrimers

	PAMAM G4		PAMAM G6	
	Low affinity	High affinity	Low affinity	High affinity
K_b [M^{-1}]	(2.6 ± 0.2) 10^5	(3.70 ± 0.34) 10^6	(1.2 ± 0.1) 10^5	(1.38 ± 0.18) 10^6
n	0.60 ± 0.11	0.34 ± 0.09	76.34 ± 4.12	22.73 ± 2.21

and n (Table I). It turned out that the amount of binding centers that are characterized by lower affinity towards ANS is always bigger (64% for PAMAM G4 and 77.1% for PAMAM G6). We can predict that one type of binding site is located deeper in the dendrimer, whereas the other is closer to the surface, or just on the surface. If it is true then it means that these two types of places of ANS location would be characterized by a different degree of hydrophobicity. The shape of ANS spectrum in the presence of dendrimers is wide enough to be a result of two spectra. To prove this, we checked the shape of synchronous spectra of ANS in the system with dendrimers (Fig. 5). Synchronous scan spectra enable to resolve two peaks near the maximum in a wide spectrum. The ANS fluorescence synchronous scan spectra had two maxima: at 350 and 390 nm that corresponded to the less and more polar environment, respectively. The ratio F_{390}/F_{350} increased with increasing concentration of ANS (Fig. 6). It indicates that first the more hydrophobic centers were filled up and then those that were close to the surface. Comparing K_b values, we can conclude that, in the case of both dendrimers, the predominant amount of ANS is located near the surface and only approximately one-third is better screened from water molecules by deeper incorporation.

It is striking how big the difference in the amount of interacting ANS molecules per one molecule of dendrimer is for PAMAM G4 and PAMAM G6 dendrimers. In the case of PAMAM G4, less than one molecule of ANS interacts with one dendrimer. Although the fourth generation is considered to be the first one that provides the globular shape of the dendrimer, it seems that the structure is still too open and too flexible to incorporate ANS. Probably, this dendrimer creates the net with ANS molecules that enables one molecule of ANS to interact with two dendrimers [Fig. 7(A)]. Therefore, ANS is surrounded by dendrimers. Similar interdendrimer interactions were postulated for the system: PAMAM dendrimers, 1-(trimethylammoniumphenyl)-6-phenyl-1,3,5 hexatriene *p*-toluenesulfonate (TMA-DPH), which is also a fluorescent probe.[15] On the contrary, PAMAM G6 dendrimers were very effective as host molecules. Besides 76 molecules that created a layer on the surface, ~ 23 molecules were placed deeper. It could be

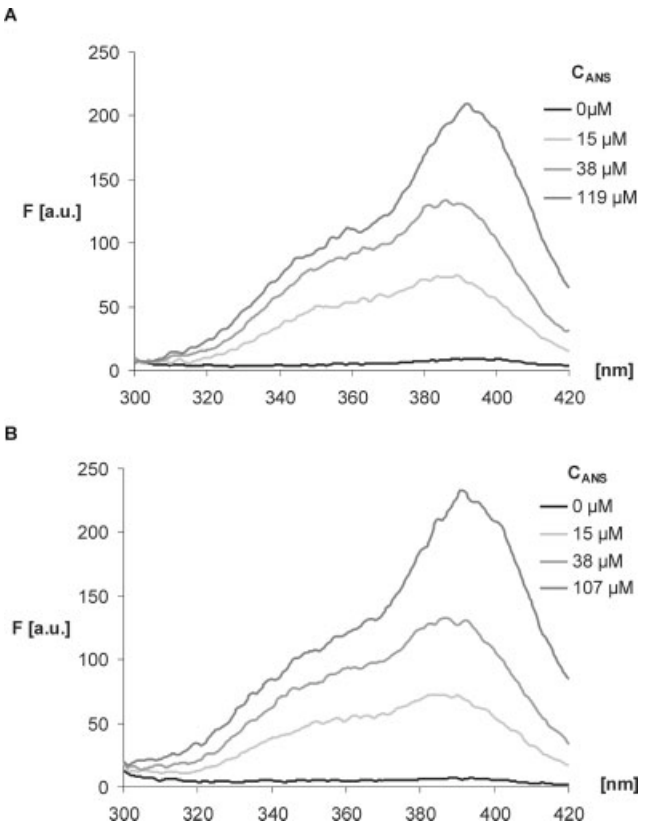

Figure 5 Synchronous spectra of ANS in the presence of 60 μM of PAMAM G4 (A) and 5 μM of PAMAM G6. $\lambda_{initial}$ = 300 nm, $\Delta\lambda$ = 122 nm, and scan speed 400 nm/min.

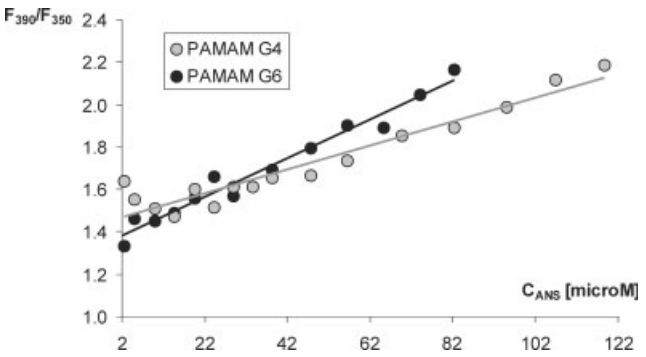

Figure 6 Changes in the ratio of fluorescence intensities at 390 and 350 nm for synchronous spectra with increasing concentration of ANS.

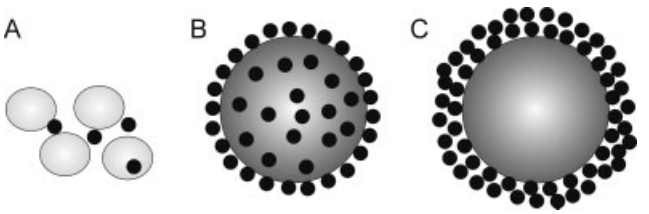

Figure 7 Location of ANS in the presence of PAMAM G4 (A) and PAMAM G6 (B and C).

either the internal cavities [Fig. 7(B)] or the inner layer on the surface that was protected from the contact with water by the outer shell of ANS molecules [Fig. 7(C)].

CONCLUSIONS

ANS can locate both inside the dendrimer and on its surface. More ANS molecules interact with the surface but the binding constants that characterize this process are always lower than the binding centers that are inside the dendrimer. To provide effective encapsulation, higher generations of dendrimers should be employed.

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