Estimation of PAMAM Dendrimers' Binding Capacity by Fluorescent Probe ANS

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Dendrimers are globular, hyperbranched polymers which possess a high concentration of surface functional groups and internal cavities. These unique features make them very useful to many biomedical applications, especially as carrier molecules. This study presents results of estimation of polyamidoamine (PAMAM) dendrimers and human serum albumin (HSA) binding capacity of fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (ANS). It has been shown that fluorescent probes can be used for quantitative analysis of dendrimers' binding capacity.

INTRODUCTION

Dendrimers are a new class of polymers with a well-defined molecular structure [1–3]. They are monodisperse, globular macromolecules. Dendrimers are synthesized in a stepwise manner from branched monomer units. In the first step branched monomers react with a polyfunctional core, in turn leaving end groups on a surface that can react again. The number of terminal groups increases after the addition of the layer of monomers. The more layers of monomers are attached, the higher generation of dendrimer is obtained.

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-OH G4 dendrimers have the same number of hydroxy groups at chain-ends. Molecular weight for PAMAM G4 and PAMAM-OH G4 equals 14215 and 14279 Da, respectively.

Due to a specific synthesis dendrimers have some interesting properties which distinguish them from classical linear polymers. Dendrimers possess many functional end groups which are responsible for high solubility and reactivity and empty internal cavities. These specific properties make dendrimers suitable for drug delivery systems. Drug molecules can either be attached to dendrimers' end groups, or encapsulated in the macromolecule interior [4,5]. Both strategies are very promising in targeted antitumor therapy. When one thinks about dendrimers as possible drug delivery agents it is important to know their ability to bind small ligands.

In living organisms the main transporting function is played by serum albumins which are the most abundant proteins in plasma (50–60% of the total amount of plasma proteins) [6–10]. Human serum albumin (HSA) is one polypeptide chain convoluted into three domains and looking like prolate ellipsoid $110 \times 38 \times 38$ Å [11] with molecular weight of 65 kDa [10]. This protein binds many endogenous and exogenous ligands and for many drugs binding to serum albumin is a critical determinant of their distribution and pharmacokinetics. In spite of negative surface charge the molecule of HSA has a high affinity to anionic ligands because of the presence of cationic

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groups and hydrophobic pocket in a structure of albumin binding centers [12,13]. The albumin capacity of binding anionic ligands was measured by different fluorescent probes (ANS, K-35, Pyrrone Red) [6–14].

Thus, it was very interesting to clear up the questions: Is it possible to apply the technique mentioned above for estimation of dendrimers' binding capacity? What is their binding capacity? To elucidate these questions was the aim of this work. In our studies we used anionic fluorescent probe ANS to examine the binding capacities of cationic PAMAM G4 and neutral PAMAM-OH G4 dendrimers and compare them with HSA.

MATERIALS AND METHODS

PAMAM and PAMAM-OH dendrimers (generation 4) were purchased from Aldrich (UK). 1-anilinonaphthalene-8-sulfonic acid (ANS) was obtained from Sigma (USA). All the other chemicals were of analytical grade. Double-distilled water was used to prepare the solutions. ANS was dissolved in dimethylsulfoxide (DMSO). For fluorescent measurements phosphate-buffered saline (PBS: 150 mmol/L NaCl, 1.9-mmol/L NaH_2PO_4, 8.1 mmol/L Na_2HPO_4, pH 7.4) was used. Fluorescence spectra were taken with a Perkin-Elmer LS-50B spectrofluorometer at room temperature (20°C). The excitation wavelength was set at 370 nm and the emission range was set between 400 and 600 nm. The excitation and emission slit widths were 5 nm and 2.5 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 molecule (n) for dendrimers and human serum albumin were determined by a double fluorometric titration technique [9,10,14]. In the first fluorometric titration of ANS increasing concentrations of the binding agent (BA) were added to constant concentration of ANS and the extreme intensity (F_{max}) of ANS fluorescence was determined. It corresponded to the state where all ANS molecules were bound by the binding agent. The extreme fluorescence intensity of ANS divided by its concentration gave the specific fluorescence intensity for the bound probe (F_{sp}):

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

where:
C_{ANS}^1—ANS concentration during the first fluorometric titration.

In the second fluorometric titration the binding agent had a constant concentration (C_{BA}). Increasing concentra-

tions of ANS (C_{ANS}) were added to the binding agent and the fluorescence intensity (F) was measured. The concentration of ANS bound by the binding agent was calculated as:

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

and concentration of free ANS as:

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

The binding constant (K_b) and the number of binding centers in solution (N) can be determined from the plot of $1/C_{ANS}^{bound}$ on the ordinate versus $1/C_{ANS}^{free}$ on the abscissa, according to the equation:

$$\frac{1}{C_{ANS}^{bound}} = \frac{1}{K_b \cdot N \cdot C_{ANS}^{free}} + \frac{1}{N}. \quad (4)$$

The initial region of the curve is a straight line. We modified the Eq. (4) by replacing N by the number of binding centers per one molecule of the binding agent (n):

$$n = \frac{N}{C_{BA}}, \quad (5)$$

where:
C_{BA}—a molar concentration of the binding agent.

Thus, the final version of Eq. (4) was:

$$\frac{C_{BA}}{C_{ANS}^{bound}} = \frac{1}{K_b \cdot n \cdot C_{ANS}^{free}} + \frac{1}{n}. \quad (6)$$

RESULTS AND DISCUSSION

The pure ANS probe in aqueous solution had a weak fluorescence in a range 400–600 nm with a maximum at 520 nm. It is a consequence of its low fluorescence yield in polar environment [15]. Adding PAMAM G4 dendrimer led both to a sharp increase in fluorescence intensity and the blue shift of the position of emission maximum (λ_{max}) (Fig. 1). The dependences of λ_{max} position and ANS fluorescence intensity upon a concentration of PAMAM G4 dendrimer are shown in Fig. 2 and Fig. 3, respectively. It is known that PAMAM dendrimers do not absorb in a spectral range 250–500 nm [16]. On the other hand, the blue shift of fluorescence emission spectra and the increase of fluorescence intensity in the presence of serum albumin are for solvatochromic fluorescent probes (i.e. ANS) the main features of their binding by albumin molecules [6–14]. Thus, we can conclude that observed results were the consequence of binding of ANS by PAMAM G4 dendrimers.
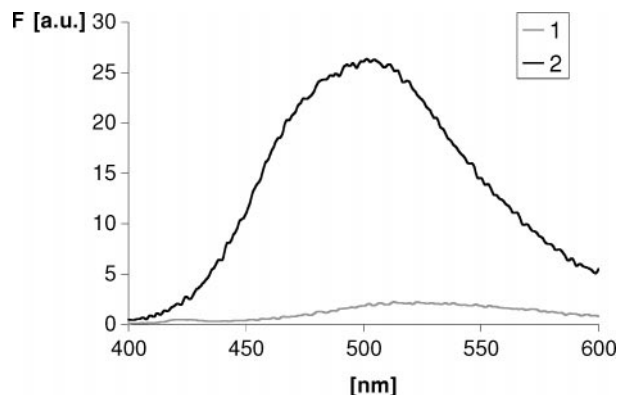
Fig. 1. Fluorescence emission spectra of ANS ($C_{ANS} = 20$ μmol/L) in the absence (**1**) and in the presence of PAMAM G4 dendrimer ($C_{PAMAM\ G4} = 105$ μmol/L) (**2**). $\lambda_{exc} = 370$ nm.
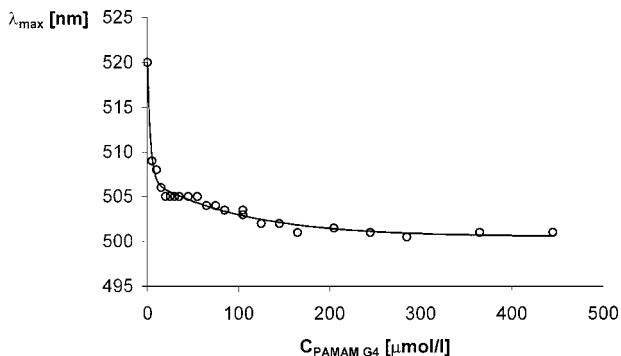

Fig. 3. The dependence of ANS fluorescence intensity on concentration of PAMAM G4 dendrimer. $C_{ANS} = 20$ μmol/L; $\lambda_{exc} = 370$ nm. (**1**)—experimental points; (**2**)—theoretical approximation of experimental points by Hill function (see text).

The binding constant (K_b) and the number of binding centers per one molecule (n) for PAMAM G4 dendrimer were determined by the double fluorometric titration method according to Eqs. (1–6). To determine the exact value of F_{max} the approximation of experimental points was made by the Hill function:
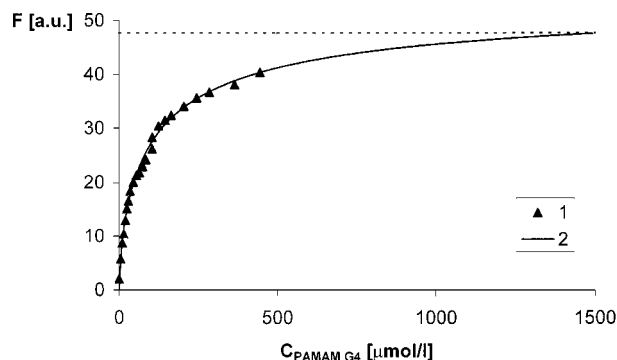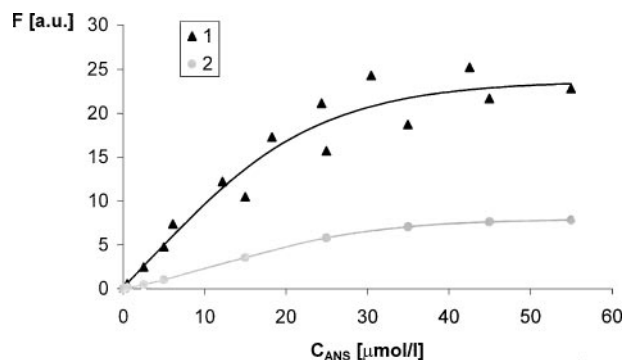
$$y = \frac{V_{max} \cdot x^m}{k^m + x^m},\tag{7}$$

where:

V_{max}, m, and k—fitting parameters, (see Fig. 3).

F_{max} was calculated as a tangent and equalled to 47.7. K_b and n for PAMAM G4 dendrimer were determined from the initial linear region of the dependence of ANS fluorescence intensity on its concentration in the presence of 50 μM PAMAM G4 dendrimer. As follows from Fig. 4, the linearity was observed for ANS concentra-

tion between 0 and 10 μM. Moreover, in this region the possible influence of free ANS fluorescence (curve 2) on bound ANS fluorescence (curve 1) was minimal. For these concentrations the plot $C_{PAMAM\ G4}/C_{ANS}^{bound}$ on the ordinate versus $1/C_{ANS}^{free}$ on the abscissa was drawn and linear regression gave the values of $K_b \sim 5.6 \times 10^4$ M^{-1} and $n \sim 0.31$ per one molecule of PAMAM G4 dendrimer (Fig. 5).

Similarly as for PAMAM G4 dendrimers, the addition of PAMAM-OH G4 dendrimers to ANS solution led both to sharp increase of ANS fluorescence intensity and to the blue shift of the position of ANS emission maximum λ_{max} (Figs. 6 and 7) which indicated binding of ANS by PAMAM-OH G4 dendrimers.

The constant of binding (K_b) and the number of binding centers per molecule (n) for PAMAM-OH G4 dendrimer were determined analogously as for PAMAM G4
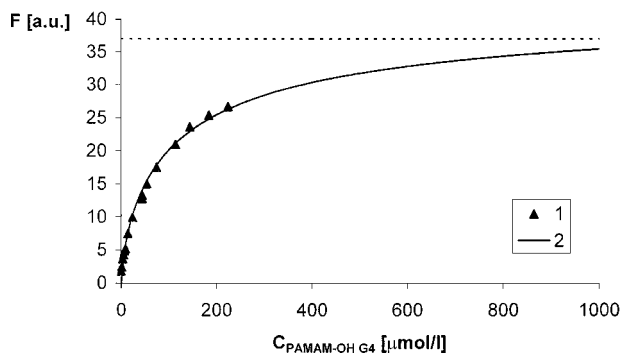

Fig. 2. The dependence of λ_{max} position of ANS fluorescence emission maximum on concentration of PAMAM G4 dendrimer. $C_{ANS} = 20$ μmol/L; $\lambda_{exc} = 370$ nm.

Fig. 4. The dependence of ANS fluorescence intensity on its concentration in the absence (**2**) and presence of PAMAM G4 dendrimer ($C_{PAMAM\ G4} = 50$ μmol/L) (**1**). $\lambda_{exc} = 370$ nm. (**1**): $\lambda_{em} = 505$ nm; (**2**): $\lambda_{em} = 520$ nm.
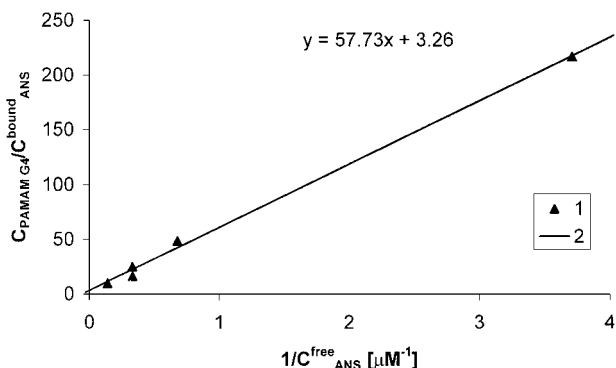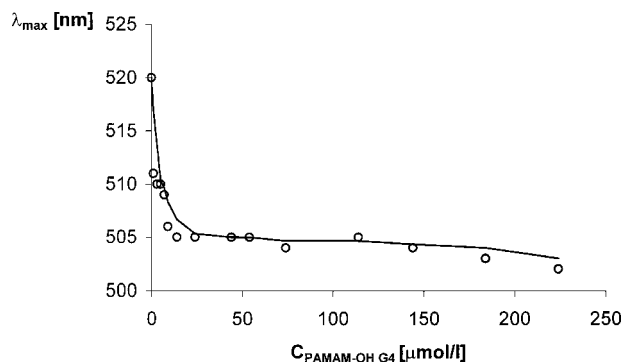
Fig. 5. The determination of K_b and n for PAMAM G4 dendrimer in the double inverse coordinates. (**1**)—experimental values; (**2**)—linear fit.

Fig. 7. The dependence of λ_{max} position of ANS fluorescence emission maximum on concentration of PAMAM G4 dendrimer. $C_{ANS} = 20 \ \mu mol/L$; $\lambda_{exc} = 370$ nm.

dendrimers. To determine F_{max} value Hill function was employed (Eq. 7) (Fig. 6). F_{max} was calculated as a tangent and came to 37. The K_b and n for PAMAM-OH G4 dendrimer were determined from the initial linear region of the dependence of ANS fluorescence intensity upon its concentration in the presence of 50 μM PAMAM-OH G4 dendrimer (Fig. 8). As is shown, the linearity and the minimal contribution of free ANS was observed in the ANS concentration range between 0 and 10 μM. The linear fit in double inverse coordinates allowed to determine the values of $K_b \sim 5.17 \times 10^4 \ M^{-1}$, $n \sim 0.32$ per one molecule of PAMAM-OH G4 dendrimer (Fig. 9).

For comparison studies, the estimation of human serum albumin binding capacity was also conducted. Figure 10 shows the influence of growing HSA concentration on ANS fluorescence intensity. The effect of ANS binding is shown in Figs. 10, 11, and 12. The constant of binding (K_b) and the number of binding centers per molecule (n) for HSA were determined by double

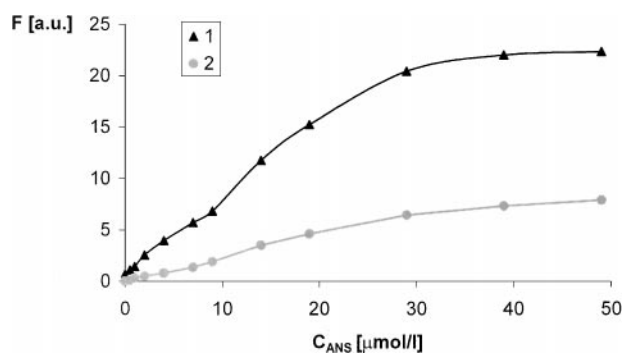fluorometric titration method according to Eqs. (1–6) for initial linear region of the dependence of ANS fluorescence intensity on its concentration in the presence of 5 μM HSA (Fig. 12). As follows from the figure, the linearity was observed for concentrations of 0–5 μM ANS. The linear fit in double inverse coordinates gave the values of $K_b \sim 1.1 \times 10^6 \ M^{-1}$ $n \sim 1.82$ per one molecule for HSA (Fig. 13).

The obtained data show that both types of dendrimers (PAMAM G4 and PAMAM-OH G4) are capable of binding ANS. Binding of ANS by dendrimer due to noncovalent forces led to a sharp increase of ANS fluorescence intensity and the blue shift of its fluorescence emission maximum. These results are in a good agreement with our previous results [17]. On the other hand, the comparison of ANS-dendrimer binding with ANS-HSA binding shows that interactions between ANS and dendrimer are significantly weaker than for the pair ANS–HSA. It was revealed by both 15–20 times bigger fluorescence

Fig. 6. The dependence of ANS fluorescence intensity on concentration of PAMAM-OH G4 dendrimer. $C_{ANS} = 20 \ \mu mol/L$; $\lambda_{exc} = 370$ nm. (**1**)—experimental points; (**2**)—theoretical approximation of experimental points by Hill function (see text).

Fig. 8. The dependence of ANS fluorescence intensity on its concentration in the absence (**2**) and presence of PAMAM-OH G4 dendrimer ($C_{PAMAM–OH G4} = 50 \ \mu mol/L$) (**1**). $\lambda_{exc} = 370$ nm. (**1**): $\lambda_{em} = 505$ nm; (**2**): $\lambda_{em} = 520$ nm.
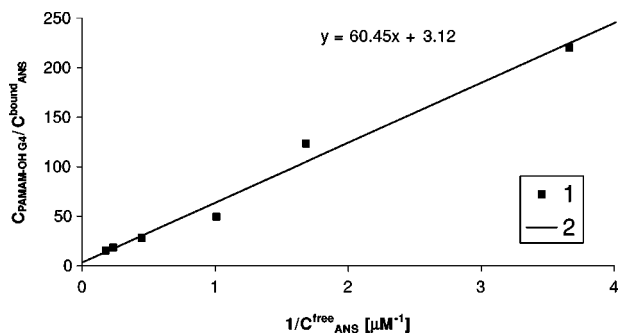
Fig. 9. The determination of K_b and n for PAMAM-OH G4 dendrimer in the double inverse coordinates. (**1**)—experimental values; (**2**)—linear fit.

Fig. 11. The dependence of λ_{max} position of ANS fluorescence emission maximum on concentration of HSA. $C_{ANS} = 10\ \mu mol/L$; $\lambda_{exc} = 370$ nm.

intensity for ANS bound by HSA than for a complex ANS-dendrimer and by a greater blue shift of ANS emission maximum λ_{max} (from 520 nm to 465 nm for ANS–HSA in comparison with the shift from 520 nm to 500–505 nm for ANS-dendrimer). The differences in binding of ANS by HSA or by dendrimer are clearly shown in their binding constants and the number of binding centers per one molecule. HSA has a binding constant 20 times bigger and 6 times more binding centers than dendrimers. It indicates that ANS has a bigger affinity toward HSA than dendrimers. This is due to differences in the size and structure between HSA and dendrimers. HSA surface possesses specific hydrophobic pockets containing cationic groups [12,13]. As a result the interactions between ANS and albumin are determined by both electrostatic and hydrophobic forces. The fourth generation of PAMAM dendrimers is characterized by a spherical shape with uniformly distributed terminal groups on the surface, but its structure is flexible. That is why it is believed that these polymers are capable of encapsulating host molecules, whereas lower dendrimer generations have open, asymmetric structure, and the higher generations (above 7) are
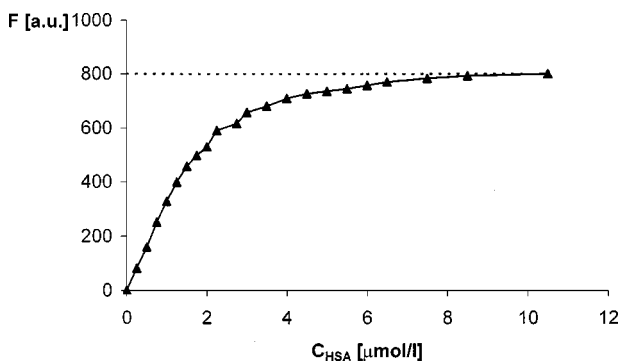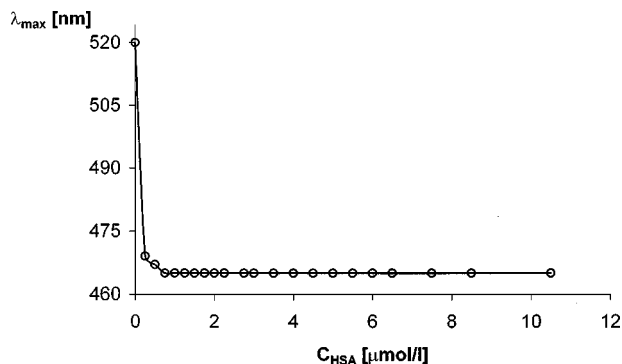
too densely packed on the surface [18]. However, our studies showed that ANS could not penetrate inside the internal, hydrophobic regions of dendrimers as it is in the case of HSA. On the other hand, enhancement of ANS fluorescence yield and a blue shift of the spectrum observed after addition of dendrimers indicate that ANS aromatic rings were placed in less hydrophilic environment. It is interesting that similar binding capacity was found for cationic PAMAM G4 dendrimers (terminal amino groups are ionized at pH 7.4) and for neutral PAMAM-OH G4 dendrimers. The earlier results have shown no incorporation effect for anionic PAMAM G3.5 dendrimers that possess carboxylate groups on their surfaces [17]. It is likely that electrostatic forces between dendrimer surface and anionic sulphonate group of ANS protect the probe from the incorporation. To conclude, the electrostatic attractions are important at the first stage of interaction but they are not the only determinants of binding capacity. Another significant difference between dendimers and HSA is their size. The molecule of dendrimer is much smaller. Its diameter

Fig. 10. The dependence of ANS fluorescence intensity on concentration of HSA. $C_{ANS} = 10\ \mu mol/L$; $\lambda_{exc} = 370$ nm.
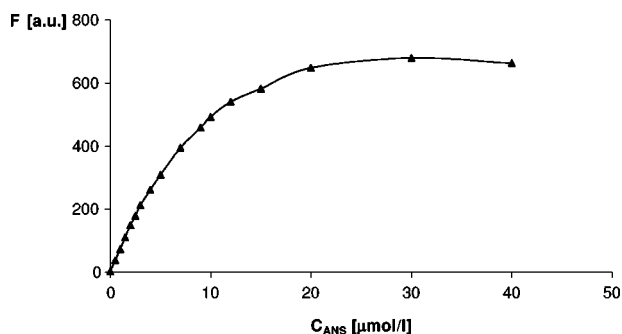

Fig. 12. The dependence of ANS fluorescence intensity on its concentration in the presence of HSA ($C_{HSA} = 5\ \mu mol/L$). $\lambda_{exc} = 370$ nm; $\lambda_{em} = 465$ nm.
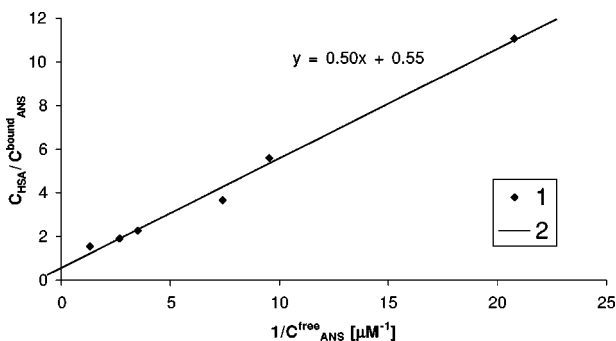
Fig. 13. The determination of K_b and n HSA in the double inverse coordinates.(**1**)—experimental values; (**2**)—linear fit.

equals $\sim$40 Å. Surface area of HSA is $S \sim 430$ nm^2 (calculations based on its dimensions [11]) whereas for the fourth generation of PAMAM dendrimers the surface is $S \sim 50$ nm^2 (calculated from a diameter [1]. Thus, it is unlikely that ANS can penetrate inside the internal hydrophobic regions of PAMAM G4 dendrimers because of its quite a big size. It means that the number of binding centers in a dendrimer increases with the enhancement of its surface area (for higher generations). Moreover, the increase of the generation number may also increase the holes inside hydrophobic regions of a dendrimer, so the fluorescent probe (ligand) can penetrate deeper into dendrimer. It should increase both n and K_b values.

CONCLUSIONS

The obtained results allowed us to draw some conclusions:

a) The technique of double fluorometric titration is applicable for the estimation of dendrimers' binding capacity. It is a fast and easy method for binding studies of fluorescent ligands which allows to determine the binding constant (K_b) and the number of binding centers (n). Thus, it is a good approach in comparative studies.

b) ANS has a lower affinity toward polyamidoamine dendrimers than for HSA. It is due to differences in the size and the surface structure between the protein and the polymer.

c) The similar K_b and n values were obtained for cationic PAMAM G4 dendrimers and for neutral PAMAM-OH G4 dendrimers. It indicates that only electrostatic forces are not responsible for interactions between ANS and dendrimers.

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