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Dendrimer Interactions with Hydrophobic Fluorescent Probes and Human Serum Albumin

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The interactions between three types of polyamidoamine dendrimers (with anionic, cationic, and neutral charge on a surface) and fluorescent dye 1-anilinonaphthalene-8-sulfonate (ANS) were studied. Double fluorimetric titration method was employed to estimate a binding constant and the number of binding centers. As fluorescent probes can serve as models of toxin molecules, dendrimers, and human serum albumin (HSA) abilities to bind ANS were compared. In the presence of HSA and dendrimers, ANS located both in HSA and in dendrimers, but the interactions between ANS and HSA were stronger.

KEY WORDS: PAMAM dendrimers; ANS; fluorescent probe; binding.

INTRODUCTION

Dendrimers are new artificial polymers topologically based on the structure of a tree. They are synthesized in a step-wise manner from branched monomer units [1,2]. One can precisely control properties of the target molecules such as shape, dimensions, density, polarity, flexibility, and solubility by choosing of these building units and functional group chemistry [3,4]. Dendrimers combine typical characteristics of small organic molecules like defined composition and monodispersivity with those of polymers such as high molecular weight that results in multitude of physical properties [1–4]. The fourth generation of polyamidoamine dendrimers (PAMAM G4), used in present paper, possesses 64 amino groups on a surface whereas PAMAM-OH G4 dendrimers have the same number of hydroxy groups at chain-ends. PAMAM G3.5 dendrimers possess 64 carboxylate groups on the surface.

Due to specific synthesis dendrimers have some interesting properties which distinguish them from classical linear polymers. Dendrimers possess empty internal cavities and many functional end groups which are responsible for high solubility and reactivity. These specific properties make dendrimers suitable for targeting, microarray systems, catalysis, and drug delivery systems [1–12]. Drugs or other molecules (guests) can either be attached to dendrimers' end groups or encapsulated in the macromolecule interior (host) [5,7–12]. One out of useful techniques for studying "host-guest complexes" is based on studying dendrimers interaction with fluorescent or EPR probes [5,7,8]. The encapsulation of a probe (eosin, pyrene, porphyrins, 2,3,6,7-tetranitrofluorenone, anthracene, etc.) showed that the size of both the guest and the dendrimer cavity determines the complex stoichiometry and that the liberation of guests can be achieved by selective removal of the protecting terminal groups [5,7–15,23–26].

In human body dendrimers can interact with components of blood and cells (i.e., proteins). That is why it is interesting to study how dendrimers interact with human serum albumin (HSA). Serum albumins are the most abundant proteins in plasma (50–60% of total amount of plasma proteins) and the main transport proteins. They bind metabolites, endogenous toxins, hormones, drugs etc. [16–22,27–35]. The detoxifying and regulatory effect of albumin is following: albumin binds the substances and

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decreases their concentration in a blood because only unbound (free) fraction of substances is physiologically active (i.e. toxic) [16–22,31]. The interaction between albumin and ligand was studied by using different fluorescent probes (ANS, TNS, K-35, etc.) [16–22]. If the binding centers of albumin are occupied by ligands the capacity of albumin to bind fluorescent probe decreases. This technique is widely used in experimental and clinical studies as a model of interaction between albumin and ligands (bilirubin, fatty acids, hormones, drugs, and herbicides) [16–20,27,30,33].

Dendrimers might also be used as detoxicants due to their ability to bind endogenous and exogenous ligands.

The aim of the present work was (1) to study interaction between PAMAM dendrimers and hydrophobic fluorescent probe ANS; (2) to study the interactions between HSA and dendrimers.

EXPERIMENTAL

Materials

Essentially-fatty-acid-free HSA, 1-anilinonaphthalene-8-sulfonic acid (ANS), dimethylsulfoxide (DMSO) were purchased from Sigma (USA). HSA was of high purity and was used without further purification. PAMAM (generation 3.5 and 4) dendrimers, PAMAM-OH dendrimer (generation 4) were obtained from Aldrich (UK). All other chemicals were of analytical grade. Water used to prepare solutions was double-distilled. ANS was dissolved in DMSO.

Fluorescence Measurements

For fluorescent measurements phosphate-buffered saline (PBS: 50 mmol/l Na-phosphate buffer, 100 mmol/l NaCl, pH 7.4) was used. Fluorescence spectra and the fluorescence synchronous scan spectra were taken with a Perkin-Elmer LS-50B spectrofluorometer at room temperature (20°C). In case of fluorescence spectra with ANS the excitation wavelength was set at 370 nm and the emission range was set between 400 and 600 nm. To control the changes in HSA during adding ANS and dendrimers the fluorescent measurements at excitation wavelength of 295 nm were made and the emission spectra were recorded from 305 to 450 nm (data not shown). In case of fluorescence 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 for all spectra were 5 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 centres per one molecule (n) for dendrimers and HSA were determined by Scatchard double fluorometric titration technique [19–21,36]. Increasing concentrations of dendrimers were added to HSA from a stock solution in PBS. Before fluorescent measurements it was checked that dendrimers were not excited by 370 nm (or 295 nm) wavelength and did not emit fluorescence.

RESULTS AND DISCUSSION

Comparative Studies on Interaction of Dendrimers and Human Serum Albumin with Hydrophobic Fluorescent Probe ANS

It is known that the pure ANS probe in aqueous solution has a weak fluorescence in a range of 400–600 nm with a maximum at 520 nm. It happens because the probes of ANS family have a very high sensitivity to small amounts of water [37–40]. The decrease of fluorescence is accompanied by a decrease of ANS lifetime what shows the dynamical nature of quenching of probes by water [41–43]. The exchange H_2O–D_2O decreases the effect of quenching by water [44–45].

Addition of HSA, PAMAM G4 dendrimer or PAMAM-OH G4 dendrimer to ANS solution led both to a sharp increase in fluorescence intensity and the blue shift of the position of emission maximum (λ_{max}) (Figs. 1–3). Adding PAMAM G3.5 dendrimer did not

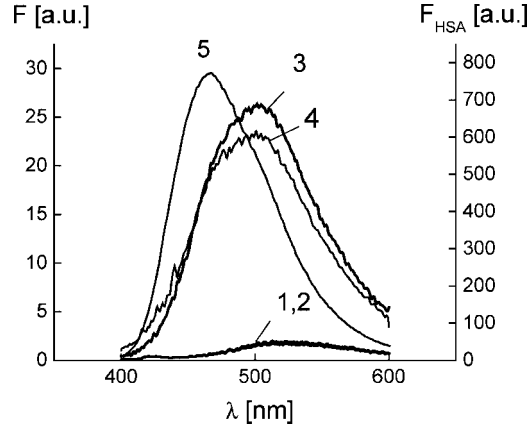

Fig. 1. Fluorescence emission spectra of pure ANS in solution (curve 1), ANS in the presence of PAMAM G3.5 dendrimer (curve 2), PAMAM G4 dendrimer (curve 3), PAMAM-OH G4 dendrimer (curve 4), ANS in presence of HSA (curve 5, right axis). $C_{PAMAMG4} = 105\ \mu M$, $C_{PAMAM\text{-}OH\ G4} = 100\ \mu M$, $C_{HSA} = 10\ \mu M$, $C_{ANS} = 10\ \mu M$, $\lambda_{ex} = 370$ nm.

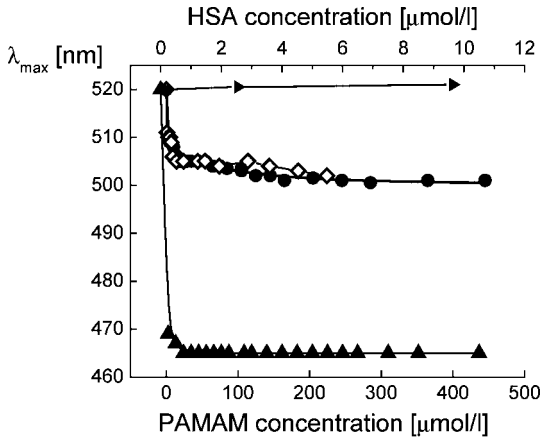

Fig. 2. The dependence of fluorescence emission maximum position of ANS on concentration of PAMAM G3.5 dendrimer (right triangles), PAMAM-OH G4 dendrimer (rhombus), PAMAM G4 dendrimer (circles) and HSA (up triangles, top axis). $C_{ANS} = 10\ \mu M$, $\lambda_{ex} = 370$ nm.

cause any effect. It is known that PAMAM dendrimers do not absorb in a spectral range of 250–500 nm [1,14,46]. Also pure HSA do not absorb in a spectral range of 310–500 nm [18,20]. On the other hand, it is known that solvatochromic fluorescent probes (i.e., ANS) undergo a blue shift of fluorescence spectra and the increase of fluorescence intensity during binding by protein or membrane. The binding occurs due to hydrophobic and/or electrostatic forces and has a noncovalent nature [18–21,37–40]. It means that observed interaction between ANS and PAMAM G4 dendrimer or PAMAM-OH G4 dendrimer may be also called 'binding.' PAMAM G3.5 dendrimer did not bind ANS probe. We can also exclude that the observed

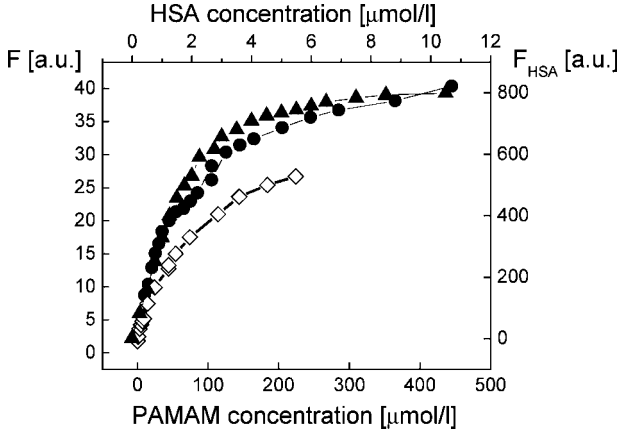

Fig. 3. The dependence of intensity of ANS fluorescence emission maximum on concentration of PAMAM-OH G4 dendrimer (rhombus), PAMAM G4 dendrimer (circles) and HSA (up triangles, right and top axes). $C_{ANS} = 10\ \mu M$, $\lambda_{ex} = 370$ nm, $\lambda_{em}^{HSA} = 465$ nm, $\lambda_{em}^{PAMAM} = 505$ nm.

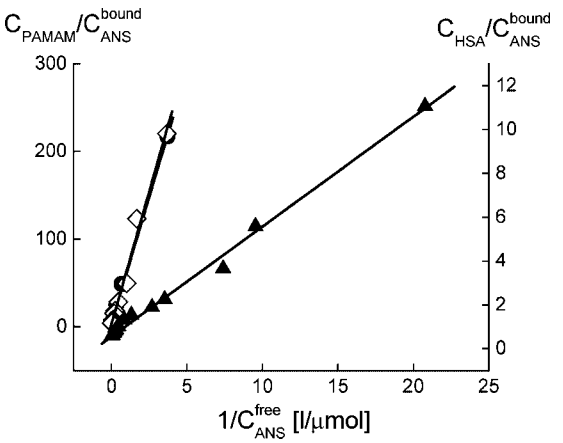

Fig. 4. The determination of K_b and n in the double inverse coordinates for PAMAM-OH G4 dendrimer (rhombus), PAMAM G4 dendrimer (circles), and HSA (up triangles, right axis).

effects are a result of disrupting ANS aggregates by PAMAM dendrimers because at low concentrations (below 10^{-4} M) ANS molecules do not aggregate [20,37–45,47].

The binding constant (K_b) and the number of binding centers per one molecule (n) for HSA and PAMAM dendrimers determined by the double fluorimetric titration method (see Fig. 4) were: for HSA—1.1×10^6 M^{-1} and 1.82 per one molecule, for PAMAM G4 dendrimer—5.6×10^4 M^{-1} and 0.31, and for PAMAM G4-OH dendrimer—5.17×10^4 $M^{-1}$and 0.32, respectively [48]. Although these data are provisional, they are useful for simple and fast quantitative analysis of interaction between probes and dendrimers.

The obtained data show that both types of dendrimers (PAMAM G4 and PAMAM-OH G4) are capable of binding ANS. 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 intensity for ANS bound by HSA than for complexes ANS-dendrimer and a greater blue shift of ANS emission maximum λ_{max} (from 520 to 465 nm for ANS-HSA in comparison with the shift from 520 to 500–505 nm for ANS-dendrimer). The differences in binding of ANS by HSA and by a 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. HSA has two binding centers of high affinity (high K_b) for ANS [18,19,22]. These two binding centers of HSA have been fully confirmed in our experiments ($n \approx 1.82$). Although in our experiments ANS has a bigger affinity toward HSA than dendrimers, the observed

differences can be easily explained by a form of used HSA. We used a very pure HSA which was additionally deprived of fatty acids that is had a maximal quantity of free binding centers. HSA which is present in blood has the binding constant 100–1,000 times less than 10^6 M^{-1} and it varies in a wide range 10^2–$10^5 M^{-1}$ depending on the state of a body [17–21,27]. In this case, the binding capacities of HSA and dendrimers are comparable.

The structures of molecules are helpful to understand differences in interaction between ANS and HSA and dendrimers. Human serum albumin (HSA) is a polypeptide chain convoluted into three domains and looking like prolate ellipsoid consisting of three domains with dimensions $110 \times 38 \times 38$ Å^3, FW of 65 kDa [49,51] and surface area ~43,000 Å^2 (calculations based on its dimensions [50]). PAMAM G4 dendrimer is a 40 Å sphere with FW of 14 kDa and surface area 5,000 Å^2 (calculated from a diameter [1–4]). 1,8-ANS looks like a planar cylinder with maximal dimensions $\sim 10 \times 8 \times 4$ Å^3 (calculations were made on the basis of its chemical structure and the length of covalent bonds [25]) but for our purpose it was considered as a sphere with a diameter of 10 Å.

HSA surface possesses specific hydrophobic pockets containing cationic groups [22,51]. The interaction of ANS with HSA may occur in two ways. The main way is when ANS penetrates into hydrophobic pockets, binds by electrostatic forces and hydrophobic interactions and the enhancement of fluorescence intensity is observed as a result of screening from water molecules. These sites of binding have the high K_b. The other way is when ANS binds to cationic groups at HSA surface by electrostatic forces only and does not fluoresce because of quenching by water molecules [52].

The fourth generation of PAMAM dendrimers is characterised by a spherical shape with uniformly distributed terminal groups on the surface, but its structure is flexible [1–4]. PAMAM G4 dendrimer has the internal cavities and they may open as a result of Brownian fluctuations of macromolecule in solution. PAMAM G4 dendrimer has 64 amino groups that is theoretically 64 binding centers. However, there are the steric limitations described by Tomalia *et al*. [53], which do not allow for binding all 64 molecules. On the basis of the Mansfield–Tomalia-Rakesh equation:

$$N = 2\pi\sqrt{(3)} \times r_1/r_2 + 1,$$

where r_1 is radius of a dendrimer and r_2 is radius of a binding molecule [53], the maximum number of bound ANS molecules is ~16. When a tight packing of ANS molecules ($r_2 = 4$ Å) is considered the maximum number of ANS molecules can be 37 in case of surface binding. This is for electrostatic packing.

However, the interaction between dendrimer and ANS may have more complex character. Dendrimers of the fourth generation are capable of encapsulating guest molecules, whereas lower dendrimer generations have open, asymmetric structure, and the higher generations (above 7) are too densely packed on the surface [1–9,54]. The enhancement of ANS fluorescence yield and a blue shift of the spectrum observed after addition of dendrimers indicate that ANS aromatic rings were placed into less hydrophilic environment. It may have occurred because of (1) full or partial penetration of ANS into dendrimer structure, (2) or/and because of surrounding of ANS molecule by dendrimers. The literature data show that electrostatic interactions may be primary in the interaction of dendrimers with liposomes [8,55–57], but hydrophobic interactions play an important role in encapsulation of small molecules [57] and small fluorescent probes [8,57–59] into dendrimers. In some cases of binding dendrimer to sodium hyaluronate [60] or fluorescent probe 2-naphthol [26] hydrogen-bonding interactions may occur.

No interactions between ANS and PAMAM G3.5 dendrimers were observed. It is likely that electrostatic forces between anionic carboxylate groups and an anionic sulphonate group of ANS protect ANS from the incorporation into dendrimer. On the other hand, the 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. It shows that the electrostatic attractions are important but not the main determinant of interactions between PAMAM dendrimers and ANS. This is probably due to the nature of ANS probe). The hydrophobic interactions are important for effective incorporation. The similar situation is observed for HSA where interactions with ANS in the sites with high K_b are fully determined by hydrophobic forces [21–22,51,61] or for cyclodextrins which enhance the ANS fluorescence by inclusion into cavities [62], or in case of interaction of fluorescent dye *Nile Red* with dendrimers [8,63]. Seemingly, several molecules of a dendrimer are needed for effective enhancement of ANS fluorescence. One molecule of dendrimer provides hydrophobic interaction—full/partial inclusion of ANS molecule into cavity (and electrostatic binding if possible) and two another dendrimer molecules provide a hydrophobic shield for ANS molecule bound to the first one.

Studying Interactions between Dendrimers and Human Serum Albumin by ANS

Figure 5 shows the changes of ANS fluorescence emission spectra at constant concentrations of ANS and HSA for different concentrations of PAMAM G4

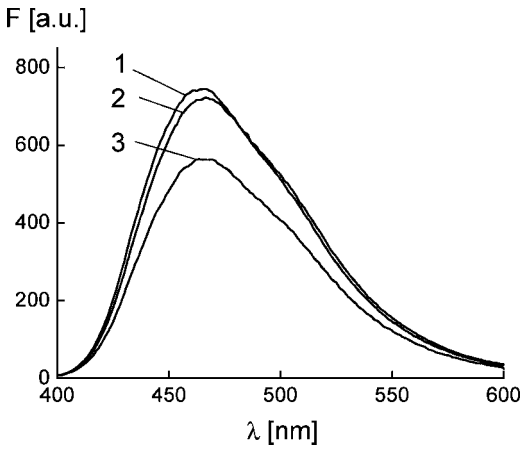

Fig. 5. The ANS fluorescence emission spectra in the presence of HSA (curve 1), the mixture of HSA with 60 μM PAMAM G4 dendrimer (curve 2) and the mixture of HSA with 360 μM PAMAM G4 dendrimer (curve 3). $C_{ANS} = 10$ μM, $C_{HSA} = 10$ μM, $\lambda_{ex} = 370$ nm.

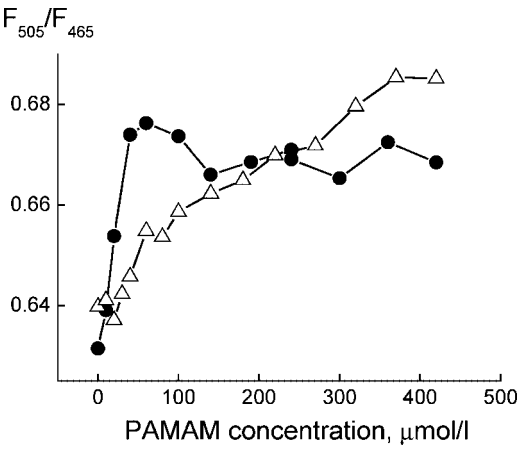

Fig. 6. Changes in ratio of intensities at 505 nm and 465 nm in ANS fluorescence emission spectra in the presence of HSA at different concentrations of PAMAM G4 dendrimer (black circles) or PAMAM-OH G4 dendrimer (white triangles). $C_{ANS} = 10$ μM, $C_{HSA} = 10$ μM, $\lambda_{ex} = 370$ nm.

dendrimer. Two effects were observed: the decrease of ANS fluorescence intensity as a result of mixture dilution by PAMAM G4 dendrimer solution as well as the redistribution of ANS fluorescence intensities at the maximum of 465 nm and a shoulder at 505 nm in ANS fluorescence spectra. The same behaviour was shown upon PAMAM-OH G4 dendrimer. It is necessary to notice that adding pure methanol in same concentrations did not induce the redistribution of ANS fluorescence maxima. ANS bound by a dendrimer has a fluorescence maximum at 505 nm. The redistribution may indicate the interactions in the system "ANS-HSA-dendrimer." For detailed analysis of these interactions the measurements of intensities ratio at 505 and 465 nm (F_{505}/F_{465}) and synchronous scan spectra of ANS in the presence of HSA at different concentrations of PAMAM G4 and PAMAM-OH G4 dendrimers were conducted. The synchronous scan spectra allow for resolving both the shoulder in the maximum and two nearby maxima in a wide spectrum. In our case ANS had two excitation maxima at 352 nm ($F = 0.88$ relatively to F_{383}) and at 383 nm ($F = 1$) when registered at 465 or 505 nm, respectively. Thus, using $\Delta\lambda = 122$ nm we obtained the maximum of excitation intensity at the emission point of 505 nm. Consequently, the ANS fluorescence synchronous scan spectra had two maxima: at 348 nm, indicating $\lambda_{ex} = 353$ nm via $\lambda_{em} = 475$ nm, and at 378 nm, indicating $\lambda_{ex} = 383$ nm via $\lambda_{em} = 505$ nm. We measured the ratio F_{378}/F_{348}. The results are presented at Fig. 6–8.

The results show that in the case when the interaction of HSA with ANS has insaturating character (ANS is added to the mixture of HSA with dendrimer), dendrimer does not affect the binding of HSA with ANS because K_b of dendrimeris of two orders of magnitude less than K_b of HSA. Nevertheless, dendrimer affects a protein molecule [46].

Another situation is observed when dendrimer is added in the presence of HSA and ANS in the molecular ratio 1:1. In this case, before addition of dendrimers practically all molecules of ANS are bound by HSA. The changes in ratios F_{505}/F_{465} and F_{378}/F_{348} may reflect at least three possible ways of interaction. First, dendrimer competes with HSA for ANS, so redistribution of maxima

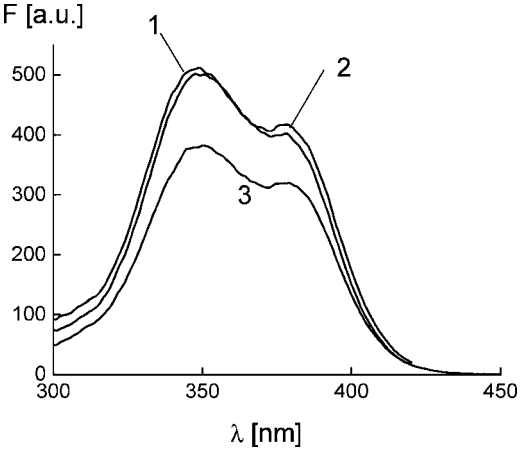

Fig. 7. The ANS fluorescence synchronous scan spectra in the presence of HSA (curve 1), the mixture HSA with 60 μM PAMAM G4 dendrimer (curve 2) and the mixture HSA with 360 μM PAMAM G4 dendrimer (curve 3). Synchronous scan from 300 to 420 nm, $\Delta\lambda = 122$ nm, $\lambda_{initial} = 300$ nm, $C_{ANS} = 10$ μM, $C_{HSA} = 10$ μM, $\lambda_{ex} = 370$ nm.

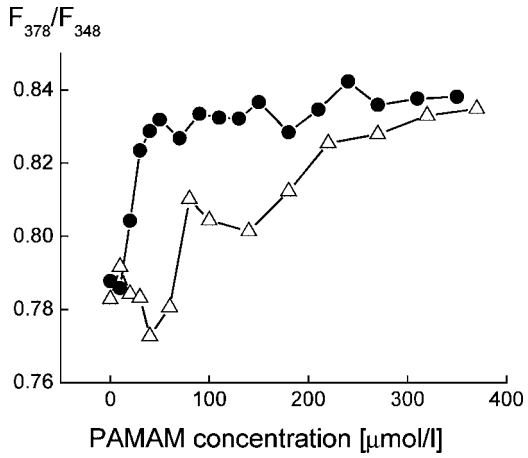

Fig. 8. Changes in ratio of intensities at 378 and 348 nm in ANS fluorescence emission spectra in the presence of HSA at different concentrations of PAMAM G4 dendrimer (black circles) and PAMAM G4-OH dendrimer (white triangles). $C_{ANS} = 10\ \mu M$, $C_{HSA} = 10\ \mu M$, $\lambda_{ex} = 370$ nm.

at 465 and 505 nm occurs due to the increase of interaction of dendrimer with ANS (see Fig. 2). Second, dendrimers affect the conformation of HSA molecule, so HSA hydrophobic pockets are partially exposed to the solvent and the maximum of ANS bound by HSA changes from 465 to 505 nm. Third, ANS may bind to cationic groups on HSA surface (the sites of HSA with low K_b) by electrostatic forces only. Then dendrimers interact with ANS (bound to HSA) screening it from water by hydrophobic forces.

The ratios F_{505}/F_{465} and F_{378}/F_{348} show the differences in the behaviour of PAMAM G4 and PAMAM-OH G4 dendrimers. The cationic PAMAM G4 dendrimer showed more effective interaction with HSA-ANS system due to the presence of anionic domains in HSA. One molecule of HSA can interact with no more then five molecules of PAMAM G4 dendrimer and after that the saturation occurs. For comparison, the neutral PAMAM-OH G4 dendrimer has weaker interaction with HSA-ANS system which corresponds to the effect observed after the addition of methanol into HSA-ANS system (i.e. decrease of polarity). The observed differences can be explained by different charge at the end groups of these dendrimers.

The similar results were obtained for interaction of cationic dendrons with bovine serum albumin [64], but those dendrons had bigger dimensions than PAMAM dendrimers and the main type of interactions was electrostatic one [64]. For sugar-persubstituted PAMAM dendrimers (which are also bigger than PAMAM dendrimers) it was shown that the interaction with bovine serum albumin had the electrostatic character and the number of interacting molecules depended on dendrimers' size [65,66]. On the other hand in triple system "dendrimer—fluorescent probe *Nile Red*—surfactant" both electrostatic and hydrophobic character have been presented [63]. The increase of the number of hydrophobic pockets as result of electrostatic interaction of dendrimer with surfactant led to dramatical increase of fluorescence of a dye [63].

Thus, the obtained results show that the technique of double fluorimetric titration is applicable for studying dendrimers and their interactions with proteins. ANS has a lower affinity toward PAMAM dendrimers than toward HSA because of differences in a size and a surface structure between the protein and the polymer. Both electrostatic and hydrophobic interactions are responsible for interactions between ANS probe and dendrimers. The interactions between ANS, HSA, and dendrimers have a complex character and dendrimers can affect binding of ANS by HSA.

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