Unusual pH-Dependent Polarity Changes in PAMAM Dendrimers: Evidence for pH-Responsive Conformational Changes

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Poly(amidoamine) (PAMAM) dendrimers have been the subject of many studies because of their novel structural properties and attractive potential applications in a variety of fields, such as drug delivery, gene therapy, and chemical separations.[1−6] PAMAM dendrimers are polyelectrolytes, and in aqueous solution can be protonated at termini (primary amines) and at branch points (tertiary amines), with the extent of protonation depending on the solution pH. In general, the protonation of polyelectrolytes strongly affects conformation; greater molecular charge results in a more expanded and more hydrated polymer conformation, as demonstrated by viscometry, light scattering, and X-ray scattering and indirectly by means of environmentally responsive fluorescence probes.[7] The pH-dependent conformational behavior of dendrimers is a subject of considerable debate, however.

We have used the polarity-responsive probe 5-(dimethylamino)-1-naphthalenesulfonic acid (DNS, **1**) to explore changes in dendrimer structure that may occur with pH titration. The fluorescence quantum yield and Stokes shift of DNS vary with solvent polarity and to a lesser extent with solvent microviscosity. Polar solvents stabilize a twisted intramolecular charge transfer (TICT) species which has a long wavelength emission; high microviscosity can slow the molecular rotation needed to form this twisted conformation.[8] The anionic sulfonate moiety of DNS can ionically bind to cationic amines, such as the terminal amines of PAMAM dendrimers, as shown below. Although the binding is weak, it is saturable; consequently, the average environment of the charged amines can be assayed through the fluorescence spectroscopy of the associated DNS probe.

Experimental Section. Polyamidoamine dendrimers were synthesized as previously described.[9] Briefly, to an ethylenediamine core, Michael addition of methyl acrylate followed by exhaustive amidation with ethylenediamine results in a four-armed "dendrimer" termed generation 0, **2**. Repetition of the sequence is used to make successively higher generations. (The dendrimer generation is equal to the number of branch points between the ethylenediamine "core" and the termini and is consequently a logarithmic measure of the molecular size.)

5-Dimethylamino-1-naphthalenesulfonic acid, **1** (99+% purity), was purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

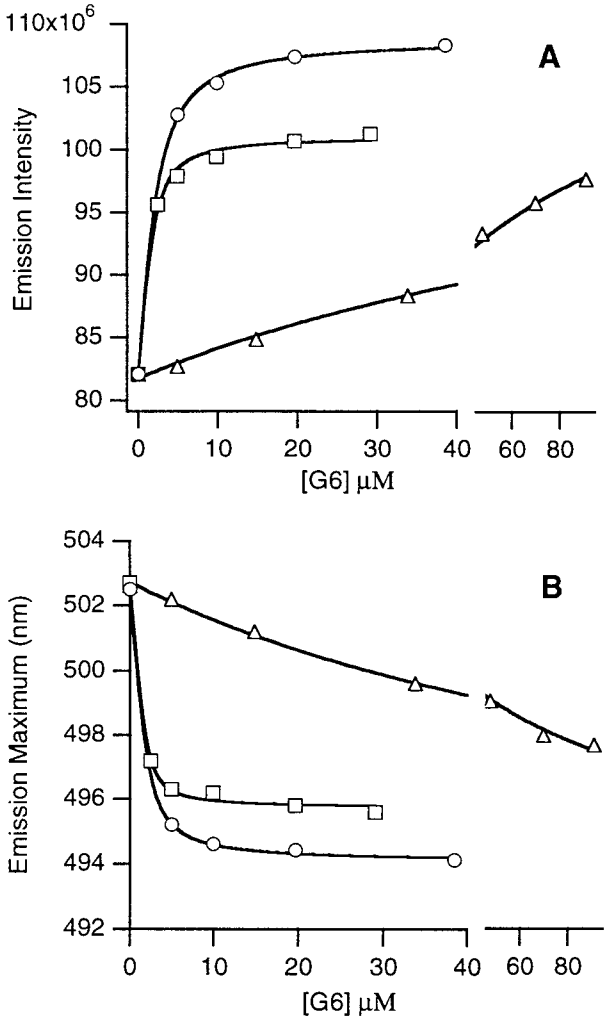
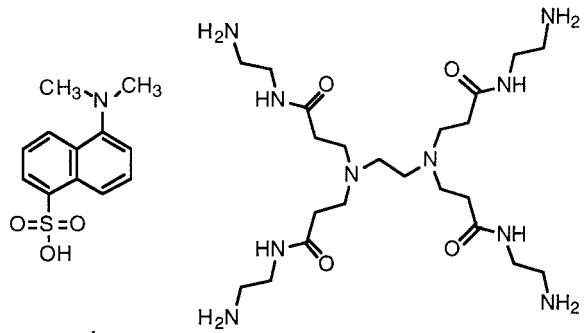


Figure 1. Emission peak intensity (A) and emission maximum wavelength (B) of 2 μM DNS with increasing amount of dendrimer G6 at different pH: pH 10 (△), pH 9 (□), and pH 8 (○). Excitation was at 310 nm. The smooth curves are fits to a simple binding equilibrium.



In all experiments, distilled deionized water was used (Millipore filtration system).

Results. The binding of DNS to PAMAM generation 6 (G6) was investigated by adding increasing amounts of dendrimers into 2 μM aqueous DNS solution at fixed pH. At pH 8 (Figure 1, squares), DNS showed an increase in the fluorescence intensity and a blue shift

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in the emission maximum, consistent with a reduced environmental polarity for dendrimer-bound DNS. The fluorescence changes saturated at ca. 20 μM G6, implying that binding of the fluorophore was complete. The increase in emission intensity and blue shift in emission maximum are then indicative of the environment of the bound dye. The maximum fluorescence increase at pH 8 was ca. 30%, while the maximum blue shift was 9 nm, indicating an environment somewhat less polar than water but considerably more polar than methanol, in which DNS emission is blue-shifted 40 nm. In the presence of 1 M NaCl, DNS did not show any significant changes in either the emission intensity or the emission maximum with similar G6 concentration (results not shown), which indicated the absence of binding at high ionic strength and confirmed the role of electrostatic interactions in binding of the negatively charged DNS to the positively charged dendrimers.

The binding of DNS to dendrimer G6 was also studied at several different solution acidities. Saturable binding was seen when the pH was between 5.5 and 10. Below pH 5.5, DNS will protonate ($pK_a = 4.5$) and become neutral, nonfluorescent, and nonbinding. Above pH 10, there are apparently very few protonated primary amines on the PAMAM dendrimer to which the anionic DNS can bind, a result confirmed by titration studies on these and other amine-terminated dendrimer molecules.[9] Even at a dendrimer concentration of 90 μM, the highest concentration examined, the DNS fluorescence intensity had not yet reached an asymptote.

Importantly, however, between pH 5.5 and pH 9.5, fluorescence changes in DNS emission are saturable, and asymptotic values of fluorescence intensity and Stokes shift will report on the local environment of the bound probes. In this pH range, when the concentration of G6 is greater than 20 μM, essentially none of the 2 μM total DNS remains free in solution. Moreover, since the concentration of dendrimer is much greater than the concentration of DNS, on average fewer than one DNS molecule will be bound to each dendrimer. Under these conditions, the bound DNS has a higher emission intensity and a smaller Stokes shift at pH 8 than at pH 9, indicating an average environment for the DNS that is less polar at pH 8 than at pH 9. Because the DNS is not covalently attached to the dendrimer, the lower polarity could reflect either the redistribution of charged amines to a less polar (e.g., more crowded) environment or the increasing ionization of amines that reside in less polar environments.

A pH titration experiment was also performed with 2 μM DNS and 91 μM G6 (Figure 2). When the pH decreased from pH 11.5, there was an increase in the emission intensity and a blue shift in the emission maximum. Significantly, when the pH was lowered below pH 8.3, the reverse effect took place, with a decrease in the emission intensity and a red shift in the emission maximum. The changes near pH 10 could be due to changes in the fraction of the bound DNS probes, since fluorophore binding was not complete at this dendrimer concentration and pH. However, from pH 9.5 to pH 5.5, the binding is complete; thus, the change in emission intensity and emission maximum must reflect changes in the local average environment of the DNS probes.

To determine whether this unusual effect could be caused by the local chemical properties of the primary and tertiary amines of PAMAM dendrimers, the fluorescence of DNS was studied in the presence of generation 2 (G2) PAMAM dendrimer. G2 dendrimer contains the same functional groups as G6 but is well below deGennes' dense-packing transition[10] that occurs at high generation. Binding of DNS to G2 is also saturable, albeit with a somewhat higher concentration of dendrimer, as expected from the presence of fewer terminal amines. The emission intensity and maximum of DNS bound to G2 show only a very weak pH dependence compared with the effects observed with G6 (Figure 2). Consequently, the much larger changes seen in G6 reflect cooperative phenomena found only in the larger dendrimer structure.

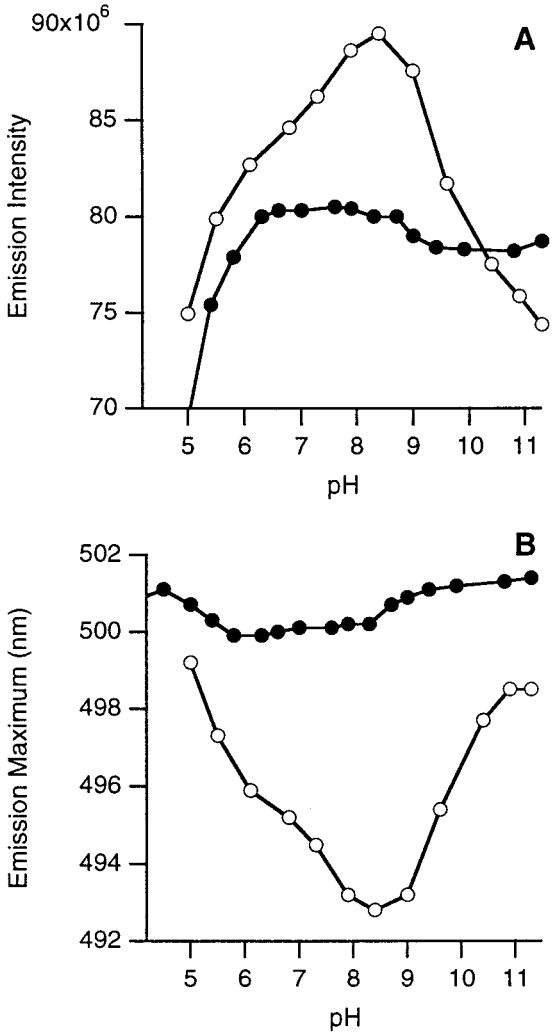

Figure 2. pH dependence of emission peak intensity (A) and emission maximum wavelength (B) of 2 μM DNS in the presence of polyamidoamine dendrimers: with 91 μM dendrimer G6 (○); with 910 μM dendrimer G2 (●). These dendrimer concentrations are sufficient to ensure complete dye binding except at pH ≥ 10. The increasing DNS fluorescence as the pH is lowered to pH 8.3 reports an environment of decreasing polarity, confirmed by the decreasing wavelength of maximum emission. The effect is nearly absent in the small, less crowded G2 dendrimer.

Discussion. The local polarity near a polymer-bound DNS fluorophore generally reflects water accessibility and, consequently, the local polymer segment density. Studies on polymer solutions have shown directly that DNS-like probes exhibit a decrease in Stokes shift and increasing emission with increasing polymer concentration.[11] The decrease in polarity (between pH 10 and 8.3) reported by DNS bound to G6 dendrimers most likely

indicates that the probe is sampling more densely packed binding sites at the lower pH. ^{13}C NMR titration experiments have shown that the primary amines at the dendritic termini have higher pK_a's than the tertiary amines at the branch points.[9] At pH > 8.3, essentially all charges are on the primary amines; since DNS binding is ionic (evidenced by the lack of binding at high ionic strength), the DNS probe is sampling the environment(s) of the primary amines. As the pH is reduced further, protonation of the tertiary amines will occur, and their environments will also be sampled.

The pH-dependent conformational behavior of dendrimers is a subject of current debate. Some theoretical models and simulations[12] have predicted that ionization of terminal groups on dendrimers should lead to a more expanded overall conformation, though with a greater crowding of terminal groups for generation 5 and above. (This result may be rationalized by comparison with the behavior of charges on a conducting sphere, which move to the surface.) In contrast, recent neutron scattering work by Nisato et al. has demonstrated that the radius of gyration of G8 polyamidoamine dendrimers is insensitive to salt and pH, which could indicate that either (a) inward-folding of G8 dendritic termini does not occur at any pH or (b) inward-folding of the same fraction of G8 termini occurs at all pHs.[13] Earlier scattering studies have shown that G7 PAMAMs have a preponderance of their end groups quite close to the periphery[14] (in deuterated methanol), in contrast to most simulations.[15−17] The authors suggest that preferential interactions of the termini with the solvent, or ionic effects, lead to this "dense shell" conformation. Other researchers have found evidence for a more uniform segment density distribution, which would require inward inward-folded termini.[18−23] Clearly, the segmental density distribution in dendrimers is still an unresolved issue.

Noncovalently bound DNS necessarily samples an average environment of the charged moieties to which it binds electrostatically. Consequently, the changes in DNS environment that we have observed could be caused by changes in the population of protonated amines, rather than by changes in the location of any particular amine. Interestingly, however, the polarity first decreases to pH 8.3, and then increases as pH is lowered further, in G6 dendrimers. In Figure 3, we present two plausible models of dendrimer behavior that could account for these results. In scheme a, some inward-folding of dendritic termini is presumed to occur at all basic pHs, resulting in a somewhat less polar dendritic interior. (Entropic driving forces will favor randomization of termini throughout the dendritic volume.) The less polar dendritic interior will disfavor protonation of inward-folded termini. At high pH (ca. pH 10) any protonation will occur on the more polar amines; as the pH is lowered to pH 8.3, the less polar amines will protonate, and the average environmental polarity reported by the DNS molecule will decrease. Further decreasing the pH begins to protonate the tertiary amines, and charge−charge repulsion causes molecular expansion, which results in an internal polarity *increase.* This last step is essential; if there is no molecular expansion, then the environment of the (internal) tertiary amines would remain at the same low polarity as that of the inward-folded primary amines. In scheme b, increasing protonation of dendritic termini is able to overcome entropy, forcing charged termini to the periphery; the resulting surface crowding results in

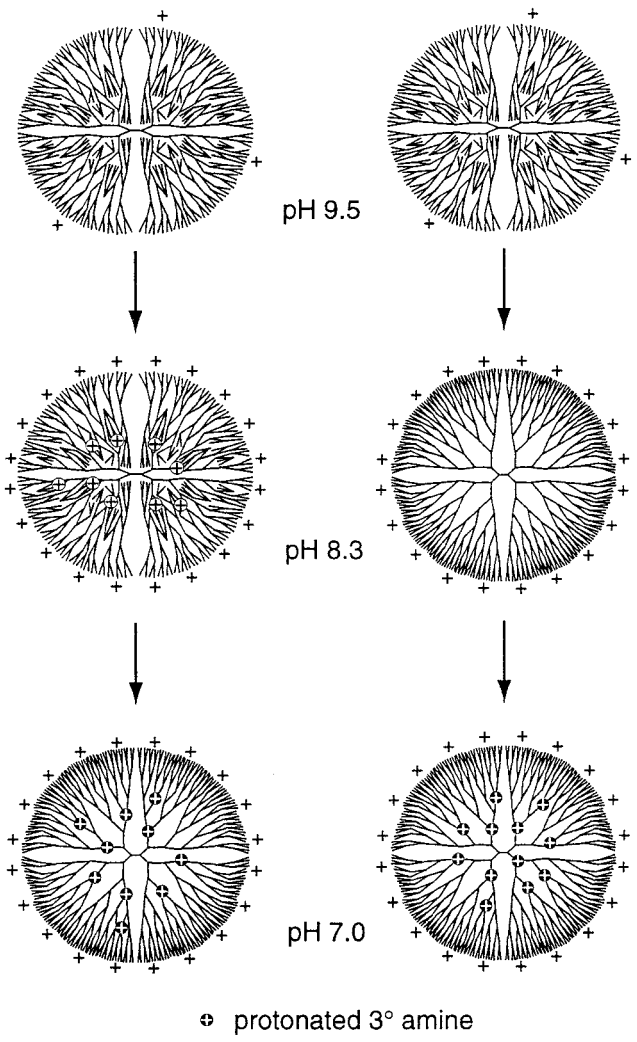

Figure 3. Scheme a: a "denser core" model. If the interior of the dendrimer is less polar than the surface, then protonation of the inward-folded termini would result in the observed polarity decrease as the pH is lowered to pH 8.3. Importantly, further lowering the pH would have to cause molecular expansion (perhaps through protonation of the tertiary amines) in order to account for the polarity *increase* below pH 8.3. Scheme b: a "denser shell" model. If protonation of the dendritic termini is able to drive them to the molecular surface, then a less polar surface region could result. Further lowering the pH would then begin to protonate the interior, more polar tertiary amines.

a lower average environmental polarity reported by DNS. In this scheme, the polarity increase on decreasing pH below pH 8.3 arises from the protonation of the tertiary amines, which are located in the less dense, more polar dendritic interior. In a nutshell, scheme a could be considered a "denser core" model of the G6 dendrimer, while scheme b is a "denser shell" model. *Importantly, both models require pH-dependent conformational changes to account for the observed polarity changes.*

This conclusion may appear to be at odds with the neutron scattering results of Nisato et al.[13] showing no changes in dendrimer size with pH variation. However, their work was performed on G8 polyamidoamine dendrimers, which are 4-fold larger in molecular weight but only 37% larger in diameter than G6 (by size-exclusion

chromatography.[24]) Consequently, G8 dendrimers have a significantly higher average density than that of G6. Interestingly, G6 dendrimers have the lowest *average* density of any generation, based on hydrodynamic measurements of molecular size.[18] The more densely packed G8 might be incapable of a charge-induced expansion. Moreover, the polarity shifts reported here are small and could be caused by relatively small shifts in segment density.

The dynamic binding between noncovalently bound DNS and the charged amines is likely a critical factor in observing these polarity changes. Covalently linking a dansyl probe to a primary amine would eliminate the positive charge on that terminus at any pH, and consequently the labeled terminus would likely behave differently from the unlabeled termini.

Finally, the measurements reported here are unable to distinguish between the "denser shell" and "denser core" models described above. There is clear evidence for a conformational change, but whether such conformational changes can be used to trigger release of physically entrapped compounds remains to be experimentally determined.

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