# Intermolecular Forces Driving Encapsulation of Small Molecules by PAMAM Dendrimers in Water

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# Intermolecular Forces Driving Encapsulation of Small Molecules by PAMAM Dendrimers in Water

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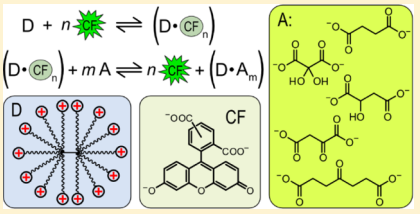
Supporting Information

**ABSTRACT:** The nature and relative strength of the intermolecular interactions responsible for the behavior of PAMAM dendrimers as encapsulating agents were investigated in neutral aqueous solution through optical spectroscopy methods. In order of relative importance, we found electrostatics, hydrogen bonding, and interactions mediated by aromatic moieties in the guest molecules to be the main drivers to complex formation in these systems. Insights were gained by studying the binding of small targeted organic anions to these dendrimers (G3−G6) by an indicator displacement method, monitoring spectral signals (absorbance, fluorescence emission, and anisotropy). This study contributes to a more complete understanding of the fundamental interactions involved when these hyperbranched polyelectrolytes function as supramolecular hosts, a role they commonly assume in many of their applications e.g. as drug delivery vectors.

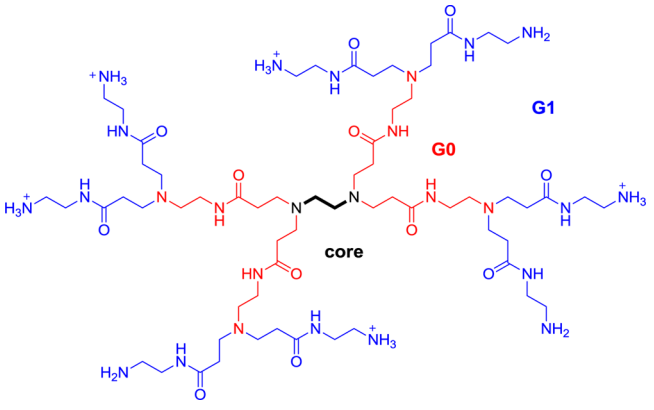



## INTRODUCTION

Poly(amidoamine) (PAMAM) dendrimers are commercially available water-soluble polymers that continue to attract attention due to their interesting structural features, such as their large globular structure,[1] the ability to control their end-group functionality,[2] and their high loading capacity.[3] These water-soluble materials are currently being explored for a wide range of applications, including gene therapy,[4] nanoreactor systems[5,6] and drug delivery.[7] In particular, amine-terminated PAMAM dendrimers are polycations in aqueous solutions near neutral pH due to the protonation of a sizable portion of the surface amine groups (Scheme 1),[8] making them good hosts for small organic molecules. Indeed, the vast majority of the current and prospective applications for these materials focuses on the ability of these structures to reversibly encapsulate and transport small organic payloads, i.e., to act as supramolecular hosts through intermolecular interactions. However, the nature of these interactions has not yet been completely elucidated, and this knowledge gap may have hindered a more widespread adoption of such interesting and highly functional dendritic structures.

Multiple groups, including ours, have used polyelectrolytes like PAMAM dendrimers in chemical recognition applications with spectroscopically active molecules.[9−11] In particular, previous studies by this group[12] and others[13−16] have reported in depth on the binding of organic dyes to positively charged dendrimers. However, monitoring the binding of optically silent species is much more challenging. We report here on a method allowing us to study the relative binding affinities of such spectroscopically silent species. This process allows us access to information as to what structural features are valuable to enhance binding to these dendritic structures, leading to an improved understanding of these interactions, in hopes that these guidelines may spur more widespread use of such polymers in molecular recognition applications.

**Scheme 1. Schematic Structure of a First-Generation (G1) Amine-Terminated Poly(amidoamine) (PAMAM) Dendrimer with a 1,2-Diaminoethane Core**[a]




[a]The conventional representation of dendrimer generations is highlighted in color. This is a much smaller structure than the ones used in the present study, and it is presented here only as an example.

## RESULTS AND DISCUSSION

The studies presented here focus on amine-terminated polyamidoamine (PAMAM) dendrimers with ethylenediamine cores of generations G3−G6. The dendrimers used in these studies are commercially available from Dendritech, Inc., in highly pure form as $H_2O$ or MeOH solutions. This range of

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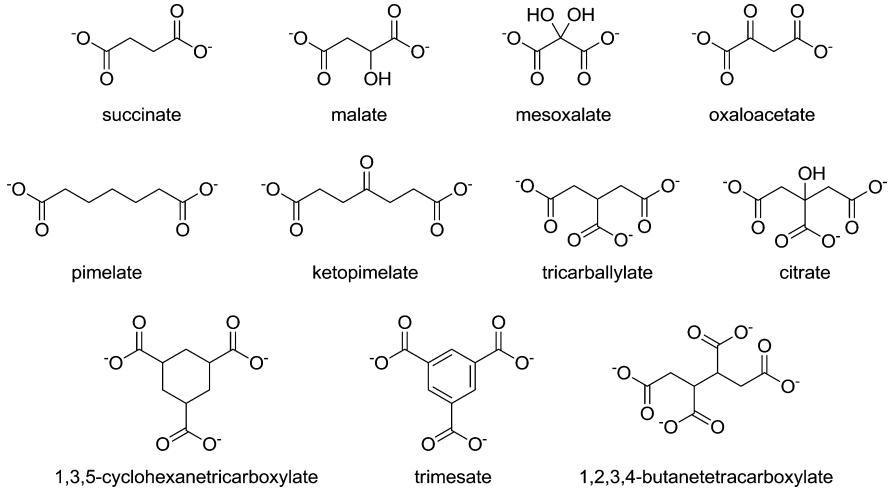
**Scheme 2. Structures of the Compounds Used as Anionic Interaction Probes in the Present Study Shown in Their Main Protonation State in $H_2O$ at pH 7.4**[a]




[a]See the Supporting Information for relevant p$K_a$ values.

**Table 1. Sets of Anionic Probes Used To Elucidate Each Intermolecular Interaction Phenomenon**

| feature under study | anionic probes used |
|---|---|
| electrostatic interactions | succinate$^{2-}$ vs tricarballylate$^{3-}$ vs butanetetracarboxylate$^{4-}$ |
| guest shape | tricarballylate (flexible) vs cyclohexanetricarboxylate (rigid) |
| guest size | succinate (short) vs pimelate (long) |
| hydrogen bonding | succinate (no OH) vs malate (1× OH) vs mesoxalate (2× OH); pimelate (no C═O) vs ketopimelate (1× C═O) (vs oxaloacetate) |
| guest steric effects | 3° OH: tricarballylate vs citrate; 2° OH: succinate vs malate |
| charge−$\pi$ interactions | cyclohexanetricarboxylate (aliphatic) vs trimesate (aromatic) |

generations was chosen for study because it represents an excellent compromise between a size large enough to provide interesting binding behavior and good loading properties. Smaller dendrimers do not display high enough affinity to be interesting for practical applications; higher generations are prohibitively expensive and seldom used in practice.

We will present here the results from binding studies conducted in aqueous solutions buffered at pH 7.4 using 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) maintained at a constant temperature of 25 °C. A number of small organic anions (Scheme 2) were selected as probes to isolate and systematically investigate the fundamental intermolecular interactions involved when these polyelectrolytes behave as supramolecular hosts in solution. As shown in Table 1, the selected anionic probes differ by one key molecular element, so that comparison of the relative binding affinities provided us insight into a range of possible intermolecular interactions.

Unfortunately, common methods to investigate such intermolecular interactions such as NMR and MS are not quite appropriate in this situation. For instance, at the higher concentration required for effective NMR studies the system's behavior may not be the same as at the much lower concentrations relevant to applications. Moreover, the most effective buffering system we have found so far (HEPES) is NMR-active, and it would swamp any signal; other common buffer systems (e.g., phosphate buffers) compete for binding to the dendritic hosts and interfere with the measurement;[9] concentrated dendrimer solutions are very viscous, and equilibration is exceedingly slow; and finally, the amount of polymeric material necessary to complete these studies would be prohibitively high. On the other hand, MS studies would certainly consume only very little sample, but the desolvation and ionization processes are likely to upset the balance of weak intermolecular interactions: although some of the limitations of MS methods have been overcome,[17] conclusions drawn from such methods may not translate to the dilute aqueous medium that we want to characterize.

We conducted our studies using optical spectroscopy, as we have done in previously reported work on similar systems.[9,12,18,18,19] We found this method to have significant advantages: it is fast, it can work directly at the very low concentration relevant to practical applications of these polyelectrolytes, and it can be automated to a great extent. However, neither the organic probes nor the dendrimers contain any significant chromophore or fluorophore through which their binding can be directly monitored, requiring us to introduce a reporter in the system. In this connection, we relied upon our previous elucidation of the binding properties of a number of dyes to positively charged PAMAM dendrimers of comparable generations (see Figure 1 for an example of such behavior).[12,18] Building upon that basis, we were able to set up a convenient dye displacement procedure[20] to monitor the binding of the spectroscopically silent anionic probes of interest.

In this approach (Scheme 3), a fluorescent indicator (5(6)-carboxyfluorescein, **CF**) is first bound to the dendrimer (**D**) to form a dendrimer−dye complex (**D**·$\mathbf{CF}_n$ in Scheme 3, top). The dendrimer:dye ratio was chosen such that all dye molecules are bound; the macroscopic optical properties of this solution are representative of the bound dye.[12,18] The nonfluorescent anionic probe of interest (**A**, see Scheme 2 for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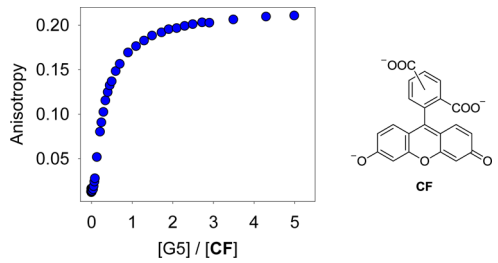


**Figure 1.** Left: isotherm describing the binding of 5(6)-carboxyfluorescein (**CF**) to a G5 PAMAM dendrimer, monitored through fluorescence anisotropy in buffered $H_2O$ (pH 7.4). [**CF**] = $2.0 \times 10^{-6}$ M. Right: structure and protonation state of **CF** when dissolved in dilute aqueous solution buffered to pH 7.4.

**Scheme 3. Dye Displacement Assay Used To Detect Binding of Spectroscopically Silent Anionic Guests A to the Dendrimers D in the Presence of the Trianionic Fluorescent Dye 5(6)-Carboxyfluorescein (CF): (Top) Dye Binding Process; (Bottom) Dye Displacement**

$$D + n\,CF \rightleftharpoons (D\cdot CF_n)$$

$$(D\cdot CF_n) + m\,A \rightleftharpoons n\,CF + (D\cdot A_m)$$

structures) is then added to displace the dye from its dendrimer complex and to form the probe–dendrimer complex, thus releasing the dye to the solution bulk, where its spectroscopic signature reverts to that typical of its free state (Scheme 3, bottom). Although neither the **A** probes nor the $\mathbf{D}\cdot\mathbf{A}_m$ dendrimer–probe complexes have any significant spectroscopic signatures, we can still monitor the complex formation process through the change in signal due to the displacement of the dye as it transitions from its bound to free state. The studies reported below were conducted by monitoring changes in the absorbance, fluorescence emission intensity, and anisotropy of these systems. The comparison of isotherm profiles obtained from such titrations of **A** probes into a solution of the dendrimer–dye complex $\mathbf{D}\cdot\mathbf{CF}_n$ provided immediate visual estimates of relative affinities which, in turn, indicated what structural features are significant for the binding to these dendritic structures and, ultimately, which intermolecular forces are at play in these systems.

The choice of the [dendrimer]/[dye] ratio to use in the displacement experiment is very important: too little dendrimer, and the dye is not fully bound at the start of the experiment, so we would not see much signal change; on the other hand, too much dendrimer, and the displacer probe will bind preferentially to the excess free host, without displacing the dye. On the basis of our previous experience,[9] we decided to work at a [dendrimer]/[dye] ratio that gave us ca. 85% binding of the dye, which afforded us good sensitivity while maintaining a wide dynamic range for the measurement.

Finally, it is important to note that stoichiometry information cannot be readily extracted from our results at this point: a combination of the low concentrations used and the complexity of the equilibria involved made it impractical, if not impossible, to determine accurate loading numbers for the small organic molecules onto the dendrimer hosts. Therefore, we did not attempt a determination of absolute binding constants; instead, we restrict ourselves here to presenting relative affinities qualitatively. Nonetheless, even simple qualitative results carry a surprising amount of valuable information about these binding interactions. After a qualitative discussion, we will also present a semiquantitative approach based on the relative amounts of probe anion necessary to cause a set degree of dye displacement (Table 2).

**Table 2. Critical Concentrations of Probe Anions Necessary To Cause 95% Displacement of the CF Dye from Its (G5·$CF_n$) Complex with the G5 PAMAM Dendrimer[a]**

| probe anion | critical concn (mM) | probe anion | critical concn (mM) |
|---|---|---|---|
| succinate | >12 | 1,2,3,4-butanetetracarboxylate | 0.04 |
| pimelate | >12 | trimesate | 0.18 |
| 4-ketopimelate | >12 | malate | 7.6 |
| tricarballylate | 1.10 | mesoxalate | 2.0 |
| citrate | 0.70 | oxaloacetate | 6.8 |
| 1,3,5-cyclohexanetricarboxylate | 1.60 | | |

[a]Lower critical concentrations correspond to higher affinity of the corresponding probe anion for the G5 dendrimer.

**Anionic Probes Targeting Specific Binding Interactions.** The major driving force for the binding of the dye molecules to the dendritic polyelectrolytes under study appears to be electrostatic interactions, as discussed below. However, careful comparison of the behavior of species carrying the same charge made it clear that other noncovalent intermolecular interactions also play an important role in determining the affinity and influencing the selectivity of these dendritic polycationic hosts. We therefore set out to study each interaction class separately and in closer detail: in particular, we will report here on the effect of overall charge, molecular structure, hydrogen bonding capabilities, and the presence of aromatic moieties in the guest molecules.

In our experiments we measured absorbance, fluorescence emission, and fluorescence anisotropy for all samples, but we will restrict ourselves to presenting only the fluorescence results for the sake of clarity, as these are fully representative of the overall trends observed. In particular, fluorescence anisotropy is a very selective reporter. Since it is only sensitive to the rate of tumbling of the fluorescent moiety in solution,[21] an increase in the fluorescence anisotropy signal is uniquely associated with the binding of the small fluorophore to the much larger dendrimers. Conversely, as the fluorophore is displaced by probe anions **A**, the anisotropy signal promptly returns to the value characteristic of the free dye, indicating the formation of a dendrimer–**A** complex.

The results presented below were obtained using fifth-generation (G5) PAMAM dendrimers, but we have carried out similar displacement studies with dendrimers of generations G3 through G6 as well. The trends obtained from other generations were comparable to those obtained with G5, so we will present only the latter results for the sake of brevity. Further data sets are included in the Supporting Information.

**Electrostatic Charge.** We investigated the effect of the electrostatic charge of the guest on the relative affinity for the dendrimer. We reported previously on the importance of this parameter in determining the affinities for the dendrimer of e.g. 5(6)-carboxyfluorescein (**CF**), a trianion in water at pH 7.4,[12] and fluorescein (**F**), a dianion in the same conditions.[18] Here we first compared the behavior of succinate, propane-1,2,3-


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tricarboxylate (tricarballylate), and 1,2,3,4-butanetetracarboxylate. These molecules respectively carry two, three, and four negative charges in $H_2O$ at pH 7.4 (see Supporting Information for relevant p$K_a$ of these and other acids used throughout this work). The results (Figure 2) show that the affinity of each

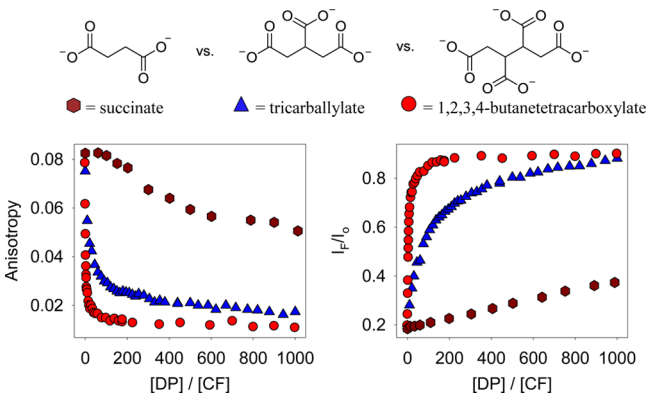


**Figure 2.** Guest charge: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with tetraanionic vs trianionic vs dianionic displacer (DP). [**CF**] = 2.0 × 10$^{-6}$ M, [G5] = 4.5 × 10$^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

guest for the G5 dendrimer increases very significantly with an increase in overall charge of the guest. The quantitative observations summarized in Table 2 show that, on average, adding one negative charge increases the relative affinity by 1 order of magnitude: this is by far the largest effect among those reported in the present study, once more highlighting the paramount importance of electrostatic interactions in these systems, even in water solutions.

**Anion Structure and Shape.** In order to explore the effect of the structure and flexibility of the guest anion on the overall affinity, we compared the binding of tricarballylate and 1,3,5-cyclohexanetricarboxylate. Both anions carry three negative charges, but the linear backbone of tricarballylate imposes few conformational restrictions, whereas cyclohexanetricarboxylate is much more rigid, with a strong preference for its triequatorial conformation. Despite that, the two anions bind to the dendrimers with comparable affinity, as shown in the displacement isotherms in Figure 3 and numerically in Table 2: their structural differences do not influence the binding significantly. We ascribe this behavior to the conformational freedom of the dendrimer: we surmise that the flexible multivalent dendrimer can accommodate virtually any spatial arrangement of charge required by its anionic guest.

Similarly, we found no difference in relative binding affinity between two linear dicarboxylates of differing chain length, succinate (four-carbon chain) and pimelate (seven-carbon chain), as shown in Figure 4. Both displacers carry two negative charges; therefore, electrostatics contribute equally to their binding to the polycationic dendrimers; any difference in steric requirements between the two anions would be reflected in their relative affinity. The fact that no difference was observed in their binding behavior further supports our hypothesis that the size and shape of the guest are essentially irrelevant in determining the guest's binding affinity for the dendrimer.

**Hydrogen Bonding.** We expected the dendrimer scaffold to take part in hydrogen bond interactions with its guests. In

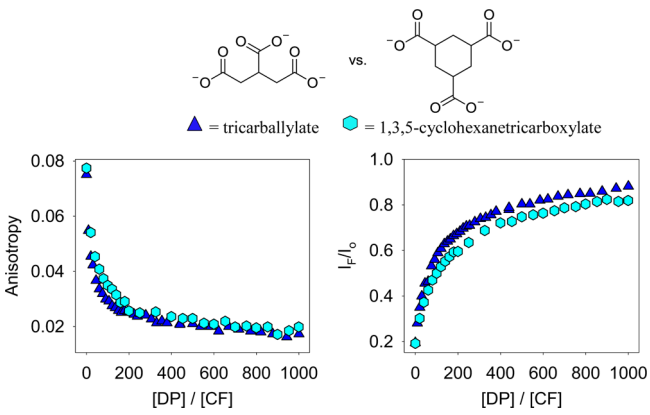


**Figure 3.** Guest shape: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with trianionic displacers with different structural features. [**CF**] = 2.0 × 10$^{-6}$ M, [G5] = 4.5 × 10$^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

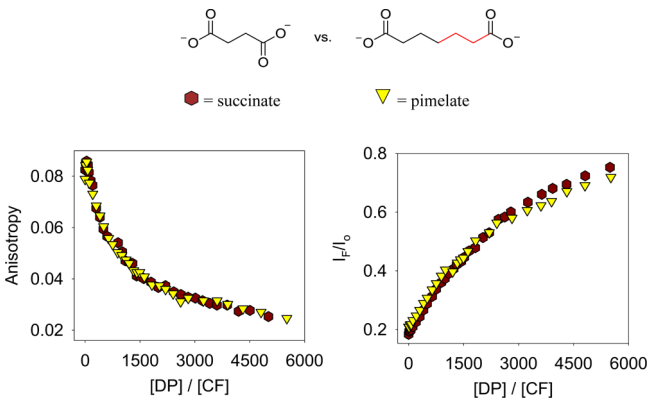


**Figure 4.** Guest size: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with displacers with different carbon chain lengths. [**CF**] = 2.0 × 10$^{-6}$ M, [G5] = 4.5 × 10$^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

fact, on the one hand the carbonyl functionalities and the nonprotonated amine groups in the dendrimer scaffold may act as hydrogen bond acceptors; on the other hand, the protonated ammonium groups on its surface may act as hydrogen bond donors. In order to test these hypotheses, we explored the two possible roles separately, using probe anions containing hydrogen bond donor and acceptor moieties.

We first investigated displacers containing a varying number of hydroxyl groups that could act as H-bond donors in order to test the behavior of the dendrimer as a H-bond acceptor. In particular, we compared the succinate, malate, and mesoxalate dianions (Figure 5). Each displacer bears the same number of negative charges, so any difference observed in their binding affinity is due to the presence of the OH groups, which in turn reports on the ability of the dendrimer to act as a H-bond acceptor. Figure 5 shows that the observed affinity increases with the number of OH groups available: malate (one OH group) binds with a higher affinity than succinate (no OH), and mesoxalate (two OH groups) has even greater affinity than malate. We conclude that the dendrimer can act as an effective hydrogen bond acceptor to establish H-bond interactions of appreciable strength with its guests. Nevertheless, the critical concentrations in Table 2 indicate that the establishment of one


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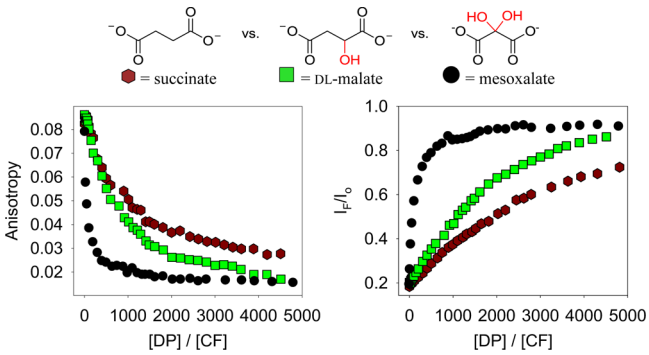


**Figure 5.** Dendrimer as H-bond acceptor: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with displacers with/without extra hydrogen bond donating groups. [**CF**] = $2.0 \times 10^{-6}$ M, [G5] = $4.5 \times 10^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

extra hydrogen bonding interaction leads approximately to a 4× increase in the apparent affinity, a smaller effect than that generated by an increase in the guest's net charge: H-bonding is a lesser contributor to overall binding affinity than electrostatic interactions.

We subsequently turned to exploring the dendrimer's capacity as a H-bond donor, using probe anions containing a carbonyl moiety acting as H-bond acceptor. In particular, we compared the binding behavior of pimelate with that of 4-ketopimelate. These two anions each bear two negative charges: any differences in binding affinity among these anions can be ascribed to the involvement of the midchain C═O group present in ketopimelate. However, no difference was detected in the observed affinities of succinate and ketopimelate (Figure 6), leading us to conclude that the dendrimer behaves as a poor H-bond donor.

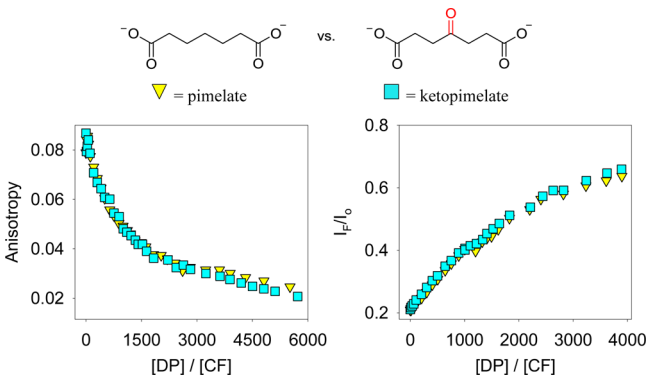


**Figure 6.** Dendrimer as H-bond donor: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with pimelate or 4-ketopimelate. [**CF**] = $2.0 \times 10^{-6}$ M, [G5] = $4.5 \times 10^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

A comparison between ketopimelate and oxaloacetate is interesting as well. In fact, both dianions formally contain an extra carbonyl moiety, so we were surprised at first to find that oxaloacetate displays a significantly higher affinity than ketopimelate (Figure 7). However, $^1$H NMR experiments in $D_2O$ indicated that the oxaloacetate anion actually exists primarily in its enol form in water solution as shown in the literature with similar carbonyl compounds,[22] due to the presence of a stabilizing intramolecular hydrogen bond

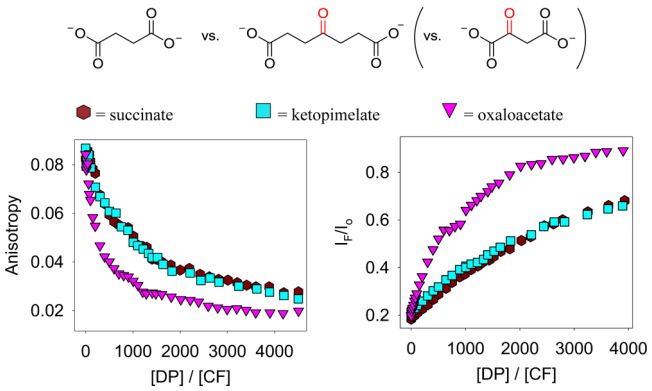


**Figure 7.** Enolizable anions: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with enolizable vs nonenolizable displacers. [**CF**] = $2.0 \times 10^{-6}$ M, [G5] = $4.5 \times 10^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

interaction (see Scheme 4). On the other hand, the corresponding ketopimelate enol cannot form such a convenient intramolecular hydrogen bond, and it exists in solution almost exclusively in the keto form (see Supporting Information for NMR spectra). Oxaloacetate is therefore more likely to behave as a hydrogen bond donor through its enol OH group rather than as an acceptor, in this respect being similar to the malate anion screened previously. In fact, the corresponding critical concentrations for 95% dye displacement (Table 2) for malate (7.6 mM) and oxaloacetate (6.8 mM) are very similar, further confirming this hypothesis.

We also tested the effects of hydrogen bonding in systems bearing three negative charges by comparing the tricarballylate and the citrate trianions, which differ only in the fact that citrate contains a hydroxyl group. On the basis of our previous findings, we expected citrate to bind with higher affinity than tricarballylate because of its extra H-bond donating group. However, the two anions were found to bind with essentially identical affinity (Figure 8). In this case, other factors are at play that prevent the OH from participating in the interaction effectively. For one, tertiary OH groups such as the one present in citrate are known to be highly sterically hindered and consequently to suffer from reduced availability of the OH group to intermolecular interactions.[23] Additionally, electrostatic interactions are much stronger for these trianionic systems than they are for the dianions discussed above, and the relative contribution of hydrogen bonding is consequently lower: in order to accommodate the dominant electrostatic interactions, citrate and tricarballylate are likely to assume a tripodal conformation that best exposes their three charged groups to the surface of the dendrimer. In such a conformation, however, the OH of citrate is forced to point away from the dendrimer, thereby removing citrate's competitive advantage.

**Effect of $\pi$-Systems.** As a final point, we probed the effect of the presence of aromatic systems in the guest species. The results of displacement titrations carried out with benzene-1,3,5-tricarboxylate (trimesate) and 1,3,5-cyclohexanetricarboxylate highlight a striking difference in the relative affinity of the two displacers for the dendritic polyelectrolyte (Figure 9). The two displacers carry the same charge and have similar size and spatial arrangement of charged groups, as shown by comparison of their estimated molecular surfaces and volumes, which are found to be within 4% and 8% of each other, respectively (see


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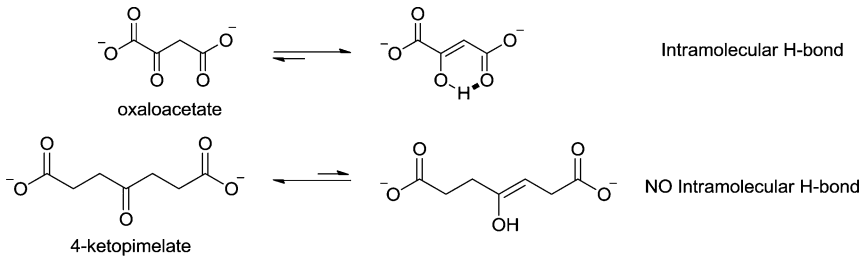
**Scheme 4. Keto−Enol Tautomerism of the Oxaloacetate and 4-Ketopimelate Anions**



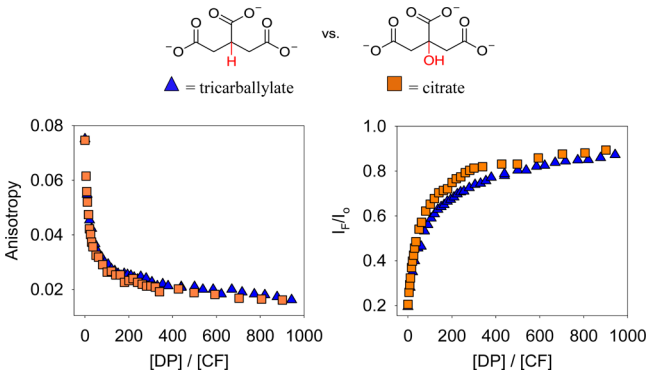




**Figure 8.** H-bonding in trianions: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with trianionic displacers with and without an OH group. [**CF**] = 2.0 × $10^{-6}$ M, [G5] = 4.5 × $10^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

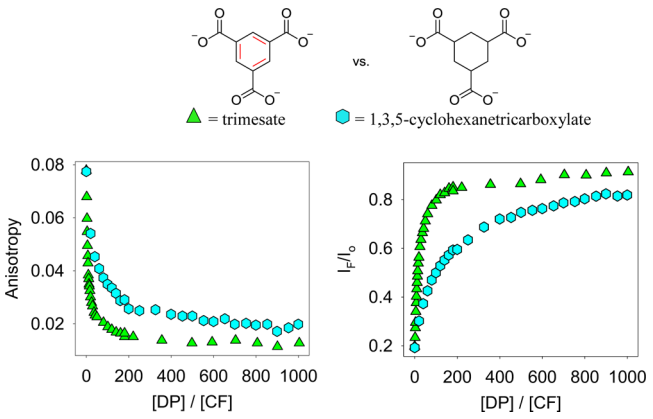
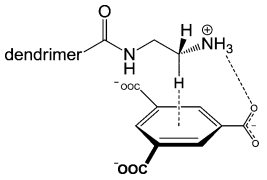



**Figure 9.** Interactions with a π-system: fluorescence intensity and anisotropy isotherms from titration of a G5·**CF**$_n$ complex with trianionic aliphatic and aromatic displacers. [**CF**] = 2.0 × $10^{-6}$ M, [G5] = 4.5 × $10^{-7}$ M in buffered $H_2O$ (50 mM HEPES at pH 7.4), $\lambda_{exc}$ = 485/20 nm, $\lambda_{em}$ = 560/40 nm.

Supporting Information for details). This consideration allows us to rule out charge density as a possible cause of the observed effect. The difference must then be ascribed to a secondary interaction that the trimesate anion alone is capable of establishing with the dendrimer scaffold. We propose that this extra contribution to the overall affinity is due to an interaction involving the aromatic core of the trimesate anion and promoted by the surface ammonium groups of the dendrimer (Scheme 5).

The positive charge of the dendrimer's ammonium groups induces significant polarization of the C−H bonds on the carbon adjacent to it in the dendrimer backbone. One of these C−H bonds is positioned ideally to interact with the aromatic

**Scheme 5. Hypothesized CH−π Interaction**[a]




[a]An ammonium group of the dendrimer interacts with the carboxylate group of trimesate; this arrangement allows the aromatic core of trimesate to interact with the electron-poor H atom belonging to the $CH_2$ group adjacent to the ammonium ion.

cloud of the guest in a CH−π interaction. The overall process is promoted by the electrostatic interaction between the ammonium cation in the dendrimer and the carboxylate anion in the guest, as described in Scheme 5.

This interaction is of course absent in the case of the cyclohexanetricarboxylate anion, which results in its lower apparent affinity (Figure 9). This is readily apparent by comparison of the electrostatic potential maps of the two molecules (Figure 10, details in the Supporting Information).

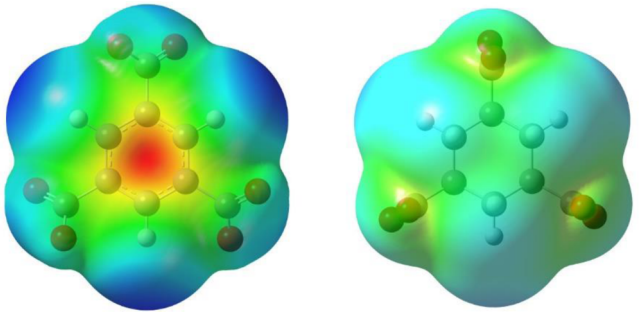


**Figure 10.** Electrostatic potential (ESP) maps for the trimesate (left) and the cyclohexanetricarboxylate (right) anions. These maps were drawn at the same mapping value boundaries; in particular, the red color represents the regions of space most attractive for a positive charge, and the blue color represents those regions least attractive to a positive charge.

The red region close to the aromatic ring in the trimesate anion is attractive for a positive charge, and it can establish the stabilizing interaction with the dendrimer's backbone discussed above; the cyclohexanetricarboxylate possesses no such region and cannot establish that interaction.

Finally, Table 2 summarizes quantitative insights into the relative affinities of the probe anions for the G5 PAMAM dendrimer. Each anion was assigned a "critical concentration", i.e., the concentration of the probe anion necessary to cause 95% displacement of the **CF** dye from its (G5·**CF**$_n$) complex, as measured from the anisotropy displacement isotherms. On this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scale, a lower critical concentration corresponds to higher binding affinity of the corresponding probe anion for the G5 dendrimer. Using these values, we can readily rank the relative importance of each intermolecular interaction we studied. For instance, increasing the negative charge on the guest by one unit brings about a more than 10-fold increase in affinity, the largest effect among those studied. Most surprisingly, the presence of an aromatic moiety in the guest also leads to a similar increase in affinity, showcased here by the comparison of cyclohexanetricarboxylate and trimesate anions. This is an unexpected outcome of this study, and it opens interesting avenues in the use of these dendrimers for selective binding to aromatic over aliphatic guests.

Furthermore, the presence of hydrogen bond donor groups (OH) in the guest has significant effects, inducing a 4-fold increase in affinity, as shown by the malate−mesoxalate comparison. Quite surprisingly, the dendrimer was found to behave as a much worse H-bond donor than it does as an acceptor, as implied by the much smaller influence of hydrogen bond acceptor groups in the guest (e.g., pimelate vs ketopimelate). Finally, shape and size of the guest have almost no effect on the affinity, thanks to the flexibility and multivalency of the dendrimer scaffold.

## ■ CONCLUSIONS

The role of various intermolecular interactions involved in the binding between polyanionic probes and PAMAM dendrimers was explored using an indicator displacement method based on simple optical spectroscopy techniques. The results of these experiments allowed us to evaluate the relative importance of various intermolecular interaction modes to the overall binding process.

We found electrostatic interactions to be the most influential by far: the overall charge of the guest is a prime predictor of relative affinity. We also demonstrated that a significant stabilizing interaction exists between the dendrimer scaffold and the electron cloud of an aromatic system. Hydrogen bonding interactions are also important: the dendrimer scaffold acts as an effective H-bond acceptor, therefore displaying higher affinity to molecules that can act as H-bond donors. Finally, shape and size of the guest have essentially no influence on the observed affinity, thanks to the high flexibility of the dendritic scaffold.

Further studies currently underway in our lab will attempt to obtain quantitative measurements of the loading capacity for these hosts—another parameter that is clearly of key importance to their practical application. Hopefully, the insights summarized here will provide valuable tools for researchers interested in using these attractive dendritic structures as molecular scaffolds and supramolecular hosts.

## ■ EXPERIMENTAL SECTION

**Materials.** Poly(amidoamine) (PAMAM) dendrimers with ethylenediamine core and primary amine termination were used in these studies. The dendrimers were manufactured by Dendritech, Inc., and purchased directly either from the manufacturer or from their distributor (Sigma-Aldrich) as MeOH solutions of varying concentrations depending on the dendrimer generation. All experiments on dendrimer generations G3 and G6 were carried out using the same dendrimer lot, whereas G4 and G5 experiments were conducted from two lots of dendrimer. In the latter case, we confirmed lot-to-lot consistency by successfully replicating a number of fluorophore interaction experiments on both lots. The G5 stock solutions in MeOH received from Sigma and used throughout this work contained ∼5% w/w dendrimer: the exact concentration depended on the specific lot and was taken into due consideration. Dendrimer solutions were stored refrigerated at 4 °C. After dilution with buffer to obtain the desired working concentration of polymers, the final solutions used for titrations contained a negligible amount of MeOH (<0.2%). The fluorescent probe, 5(6)-carboxyfluorescein (as a mixture of isomers), was purchased from Sigma-Aldrich and used as received. Displacer anion solutions were prepared from DL-malic acid, oxaloacetic acid, pimelic acid, sodium mesoxalate monohydrate, succinic acid, and trimesic acid (from Sigma), tricarballylic acid and 1,2,3,4-butanetetracarboxylic acid (Alfa Aesar), citric acid (Fisher Scientific), 1,3,5-cyclohexanetricarboxylic acid (TCI America), and 4-ketopimelic acid (Acros). All reagents were used as received.

**Instrumentation.** Instrumental parameters used for benchtop instruments (HP 8452A diode array spectrophotometer, ISS PC1 spectrofluorometer) and for the microwell plate reader (BioTek Synergy 2) are described in detail in the Supporting Information. We used Greiner BioOne untreated (medium binding) black polystyrene 96-well microplates with optically clear bottom. The total volume in each well was 300 μL. Plates were measured immediately after preparation; no seal was applied since the aqueous solution did not evaporate appreciably during the measurement time.

**Titration Conditions.** All experiments were carried out in aqueous solutions buffered to pH 7.4 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 50 mM) using a calibrate glass pH combined electrode. The pH of the working solutions was adjusted prior to use by addition of NaOH or HCl solutions and spot-checked during a titration to make sure that it had not drifted away from the desired value of 7.4. Drift was generally not a problem, but significant adjustments had to be made when preparing solutions of anions from their corresponding acids.

**General Displacement Titration Protocol.** Stock solutions of dye, dendrimers, and displacer anions in buffered $H_2O$ were used as starting points for the titrations. All solutions used in binding experiments were made by dilution of aliquots of these stock solutions. Displacement experiments were carried out using two separate working solutions: a "titrant" and a "cuvette" solution. Titrant and cuvette solutions were made fresh for each experiment. A cuvette solution contained both the dye ($[\mathbf{CF}] = 2.0 \times 10^{-6}$ M) and the G5 PAMAM polyelectrolyte ($[G5] = 4.5 \times 10^{-7}$ M) at the appropriate ratio to form the desired bound dye complex ($G5{\cdot}\mathbf{CF}_n$). The titrant solution contained dye and dendrimer at the same concentration as the cuvette solution, and it also contained a displacer anion at an appropriate concentration to carry out the titration. Titrations on benchtop instruments were carried out by addition of multiple aliquots of titrant solution to a constant volume of cuvette solution. Using this method, the concentration of dye and dendrimer remained constant throughout the titration and dilution artifacts were eliminated.

**Multiwell Plate Experiments.** Each point in a titration profile obtained in a multiwell plate corresponded to a set of wells on the plate (normally two or three replicates). All points in the titration were constructed from appropriate "titrant" and "cuvette" solutions (see Supporting Information), laid out on a single plate, and measured at the same time. Multiple experimental parameters (e.g., absorbance, fluorescence, and anisotropy) were measured on the same plate, ensuring greater internal consistency and a significant speedup in data acquisition. Absorbance and fluorescence emission raw reads were blanked by subtracting the corresponding reading for the buffer. Replicate data points on a multiwell plate were averaged: the value reported for a titration point was the average of at least two readings. The resulting data were plotted as a function of the [displacer]/[fluorophore] ratio to produce binding isotherms. Results obtained from the plate reader were in excellent agreement with those obtained on the standard benchtop instruments.

## ■ ASSOCIATED CONTENT

### Supporting Information

Detailed experimental conditions for displacement experiments; displacement experiments on G3, G4, and G6


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PAMAM generations not shown explicitly in the main paper; H NMR spectra of 4-ketopimelate and oxaloacetate anions; p$K_a$ of relevant acids; details about QSAR estimation of molecular surfaces and volumes; studies on the kinetics of binding and displacement. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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