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Comparison of Facially Amphiphilic Biaryl Dendrimers with Classical Amphiphilic Ones Using Protein Surface Recognition as the Tool

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Abstract: Facially amphiphilic biaryl dendrimers are compared with the more classical benzyl ether amphiphilic dendrimers for molecular recognition, using protein binding as the probe. The protein used for the proposed study is chymotrypsin (ChT). A generation-dependent binding affinity was observed with the benzyl ether dendrimers, while the affinities were independent of generation in the case of the biaryl dendrimers. Similarly, although the ligands incorporated in both dendrons are the same, the biaryl dendrimers are able to bind more proteins compared to the benzyl ether dendrimers. For example, G3-dendron of biaryl dendrimer can bind six molecules of chymotrypsin, whereas G3-analogue of benzyl ether dendrimers can bind only three molecules of chymotrypsin. This result is consistent with our hypothesis that the internal layers of the facially amphiphilic biaryl dendrons are solvent-exposed and accessible for recognition. In addition, the systematic size differences in dendrons were also used to gain insights into the substrate selectivity that the enzyme gains upon binding to a ligand scaffold.

Introduction

The globular shape of dendrimers combined with the fact that these molecules can be obtained with a high degree of control in molecular weight have made them attractive candidates for supramolecular chemistry.[1,2] One of the major advantages that dendrimers provide is the ability to display multiple copies of ligand functionalities to bind to a receptor, which takes advantage of features such as polyvalent interactions.[3] In cases where such a polyvalency is investigated, it is only the peripheries of the dendrimers that are decorated with the ligand functionalities.[4] This is mainly because it is only the peripheral functionalities that are thought to be fully solvent-exposed and therefore can make available multiple copies of a ligand for recognition. We have recently reported an amphiphilic dendrimer in which every repeat unit within the dendrimer backbone contains a hydrophilic and a hydrophobic functionality.[5] We had suggested that, in contrast to the classical amphiphilic dendrimers,[6−8] the biaryl ones adopt a conformation

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in such a way that *all* the hydrophilic functionalities throughout the molecule's backbone are presented on the solvent-exposed exterior in water. In such a conformation, it is thought that all the hydrophobic functionalities are presented in the interior of the assembly. If this were the case, the number of possible ligand functionalities that can be presented within a dendrimer backbone is nearly twice as much as that of the classical dendrimers, within a generation. For example in a G-3 dendron, while one could have 8 copies of a ligand in the classical dendrimers, it is possible to have 15 copies of the ligand in our dendrimers, if our structural hypothesis is correct. Could we then use a ligand−protein interaction as a probe to test this structural hypothesis? We address this issue by estimating the binding ratio of these two classes of dendrimers with the protein, α-chymotrypsin (ChT). Similarly, using dynamic light scattering studies, we have previously shown that our biaryl dendrimers aggregate to form particles ranging from 10 to 40 nm. Thus, a large number of ligand functionalities are preorganized on the surface of the particle in the biaryl dendrimers in solution. On the other hand, the Fréchet-type classical amphiphilic dendrimers do not form such aggregates (vide infra). Therefore, in the latter case, there is a systematic, incremental change in the number of ligand functionalities presented on a unimolecular dendrimer surface in the latter case. By comparing the two scaffolds, it is interesting to ask what is the critical size needed for an efficient binding that compares to a scaffold that contains numerous ligands on a 10−40 nm particle? This paper concerns the comparison of our biaryl-based amphiphilic dendrimers with the classical Fréchet-type benzyl ether amphiphilic dendrimers[8a,9] to gain insights into such structural requirements for recognition.

Simple anionic functionalities such as carboxylic acids have been previously used to recognize the cationic patch of ChT that surrounds the active site of the protein.[10] In our own previous work, we had used amphiphilic homopolymers containing carboxylate units to bind to ChT. In that work, our objective was to identify the effect of the polymer−protein interactions upon the structure and function of the enzyme. This work by us and related work by others have established that poly-carboxylate scaffolds are effective in binding and modifying the enzymatic activity of ChT. In the present work with dendrimers: (*i*) We utilize the binding ability to protein surfaces as a probe to understand and compare the conformational features of two classes of dendrimers. That is, others and we have previously used artificial molecular scaffolds to influence the properties of a biological macromolecule. In the present case, we use the influence of binding on the behavior of the biological macromolecule to understand the properties of the artificial scaffold. (*ii*) Since we can increase the number of ligands highly systematically in the fully monodisperse dendrimer scaffold, the molecular level details of understanding the poly(ligand)−protein interactions become better. For this purpose, we compare the results from the two dendritic scaffolds with linear polymers, whenever appropriate.

Structures of both our facially amphiphilic dendrimers **1**−**4**, and the Fréchet-type benzyl ether dendrimers **5**−**7** are shown in Chart 1. Syntheses and characterization of all new molecules are available in the Supporting Information. Note that the biaryl dendrimers are based on arylalkyl ether connectivity, and therefore the benzyl ether dendrimers are appropriate scaffolds for comparison.

Results and Discussion

We first estimated the binding affinity and the binding ratio of dendrons vs chymotrypsin using the enzymatic activity of the protein against chromogenic substrates such as **S1**. The enzyme cleaves the amide bond at the C-terminal of phenylalanine in **S1** to afford *p*-nitroaniline, which can be monitored using its distinct absorption spectrum at 405 nm. Note that the determination of binding affinities and binding ratios were achieved using well-established protocols for chymotrypsin with such substrates.[10i,11] Change in enzymatic activity upon binding to the dendrimer was determined by adding an **S1** stock solution to a preincubated ChT−dendrimer solution. The experiments were carried out with different concentrations of dendrimer, while the ChT concentration was kept constant at 3.2 μM. A control experiment was performed under identical conditions without addition of dendrimer. The binding constant and ratio of the ChT/dendron were obtained by plotting the activity of ChT against dendron concentrations. No inhibition effect was observed with the small molecule G0 dendron (**1**) (Figure 1). The activity of ChT decreased significantly with **2**, with 94% inhibition observed at 48 μM and then saturated at higher concentrations. Dendrimers **3** and **4** exhibited 96% inhibition at 9.6 μM and 1.9 μM concentrations, respectively (Figure 1). The concentration of dendrons used in this study is well above the critical micellar concentration (cmc). The binding ratios obtained for each of these dendrons are also shown in Table 1. Since the binding obtained here is based on electrostatic interactions (vide infra), we have also qualitatively estimated binding ratios using gel electrophoresis, the results of which were consistent with the enzymatic assay.

The binding ratio of the dendrimer to protein might seem high at first sight. For example, the G-3 dendron **7** binds to the protein in a 1:3.3 ratio. The size of a G-3 dendron is about 2−3 nm, while the size of ChT itself is about 4 nm. Arranging three 4 nm particles three-dimensionally around another 2−3 nm particle with only a few contact points is plausible. First we will compare the binding ratios between the two classes of dendrons to investigate whether the internal carboxylates in the biaryl dendrons are available for recognition with a macromolecular receptor such as ChT. The binding ratios of dendrons **2** and **5** with ChT are 0.7 and 0.4, respectively, as shown in Table

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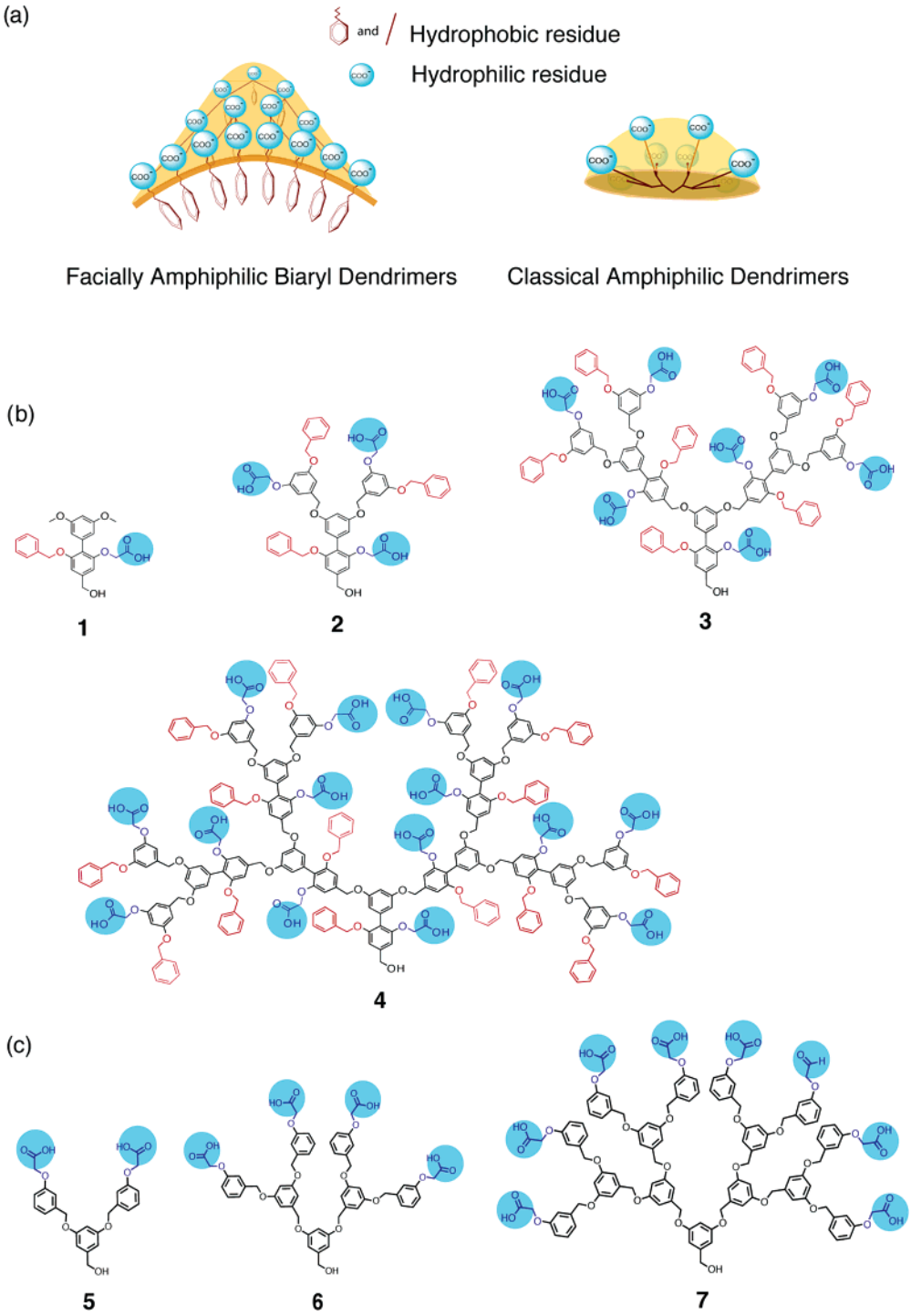
Chart 1 [a]



[a] (a) Schematic representation of facially amphiphilic biaryl dendrimers and classical amphiphilic dendrimers of G3-generation; (b) chemical structures of amphiphilic biaryl dendrimers **1**−**4**; (c) chemical structures of classical amphiphilic dendrimers **5**−**7**

1. Similarly, at higher generations, the biaryl dendrimers **3** and **4** bind more ChT molecules relative to the dendrimer **6** and **7**. This could be simply explained by the fact that the number of carboxylic units presented in the biaryl dendrons **3** and **4** is higher than that in the dendrons **6** and **7**. To further illustrate this feature, we have tabulated the number of carboxylic acid groups involved per ChT molecule with each of the dendrons. The trend among the biaryl dendrons **2**−**4** is very similar to that observed with the benzyl ether dendrons **5**−**7**. Note that we have taken in to account the internal carboxylic acid moieties in addition to the peripheral functionalities in this estimate. Therefore, this result is taken to suggest that the carboxylates in internal layers of dendrimer are available for recognition. We also find that the number of carboxylic acid groups that are needed for binding ChT decreases with generation in both dendrons. The reason for this so-called dendritic effect is not clear to us at this time.

The binding affinities of **2**−**4** toward ChT are also shown in Table 1. First of all, it is clear that a critical number of covalently tethered carboxylic acid functionalities is needed for any

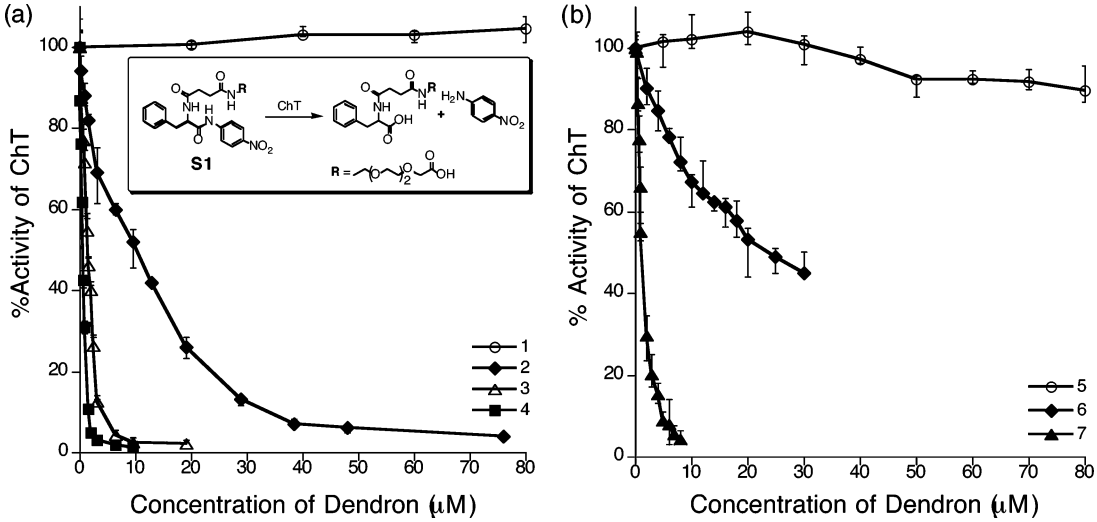


Figure 1. Inhibition study: Concentration dependent assay studies of ChT (3.2 μM); (a) amphiphilic biaryl dendrimers and (b) classical dendrimers. Assay studies were carried out with various concentrations of dendron in the presence of 5 mM sodium phophate buffer pH 7.4. The solutions were incubated for 1 h. The percentage activity of ChT was plotted against the concentration of dendron (μM).

Table 1. Comparison of Number of Carboxylic Group, Biding Ratio, and Binding Constant of Biaryl Dendrimers and Classical Dendrimers

compd	no. of carboxylic acid groups	binding ratio (no. of ChT per dendron)	no. of carboxylic groups per ChT	dissociation constant K_d (M)
1	1	n/a	n/a	n/a
2	3	0.7	4.4	5.78×10^{-6}
3	7	2.0	3.5	1.47×10^{-6}
4	15	5.7	2.6	6.02×10^{-7}
5	2	0.4	5.0	2.03×10^{-2}
6	4	1.2	3.3	1.81×10^{-5}
7	8	3.3	2.4	3.61×10^{-6}

significant binding of the protein. The biaryl **1** building block unit which contains only one carboxylic acid functionality did not exhibit any binding with the protein. Dendron **5**, which contains two carboxylic acid units, seems to be capable of binding ChT, but with a very low binding affinity. On the other hand, dendron **2** that contains three carboxylic acid groups shows a micromolar binding affinity.

Although the difference in binding affinities is quite large among **1**, **2**, and **5**, the differences in binding affinities among dendrons **2**−**4** are relatively small. This could be due to the following feature of the biaryl dendrimers. We have shown that amphiphilic biaryl dendrimers show aggregation in water to form a micelle-type assembly approximately 10−40 nm in size. Therefore, a large number of carboxylic units are *preorganized* and presented to the protein. Once it is assembled into such a nanoparticle, it is likely that the protein does not show any difference in binding affinity with generation. For example, when comparing **2** and **5**, both dendrons bind ChT with a binding ratio that requires more than one dendron per protein. But, the binding affinity of **2** is much greater, since the carboxylates are preorganized due to aggregation. Also, if our hypothesis was true, generation dependence would be observed with the Fréchet-type dendrons **5**−**7**, since these molecules do not aggregate and therefore do not preorganize any more ligands through aggregation. Indeed, generation dependence on binding affinity was observed with **5**−**7**, as shown in Table 1. We have performed DLS studies on an aqueous solution of dendrons **5**−**7** to confirm that these molecules do not aggregate. These dendrons do not exhibit any evidence of aggregation.

With the slow increments in the preorganized carboxylate ligands in the nonaggregated Fréchet-type dendrons, we have the opportunity to ask the following: what is the critical number of carboxylate moieties that need to be preorganized in a ligand scaffold to recognize the surface of ChT with a micromolar binding affinity? That is, which of the Fréchet-type dendrimers matches the K_d observed with the particles formed from dendrimers **2**−**4**? It is important to point out that the micromolar binding affinity was also observed with the recently reported polymer nanoparticles.[11] Therefore, it is reasonable to assume this binding affinity as the ceiling for these types of molecules. The G-1 dendron, **5**, compared to **2** has a lower binding affinity toward ChT. One could attribute this to the need for a critical number of carboxylate groups that are needed for the binding. However, note that, although the G-2 dendron **6** (containing four carboxylate units) exhibits much higher affinity than dendron **5**, it is still much lower than our that of G-1 dendron **2** (containing three carboxylate units). Dendron **7**, with eight carboxylate units, seems to approach the micromolar binding affinities of **2**−**4**. This result suggests that this dendron represents the size and number of ligands at which the binding affinity reaches a maximum and then plateaus.

It is also interesting to compare the present results with our previous results obtained with an amphiphilic homopolymer. The binding affinity of dendron **4** is the same as that of the amphiphilic homopolymer (7×10^{-7} M). However, the binding ratio is higher with the polymer (1:10, polymer/ChT) than that of dendron **4**. It should also be taken into account that each polymer chain contains approximately 74 carboxylic acid units (degree of polymerization (DP) is 74). Therefore, if we compare the binding ratio with respect to the number of carboxylic acid units, the number of carboxylates required to bind ChT in dendrimers is lesser than that in the linear polymer. These results could be due to the inherent difference in shape and conformational nature of the two macromolecules. This comparison further illustrates the availability of the internal carboxylate moieties for binding in the biaryl dendrimers.

Nature of Dendrimer−**Protein Interaction.** When studying the interaction of an artificial molecular scaffold with protein, it is important to understand the consequence of the binding

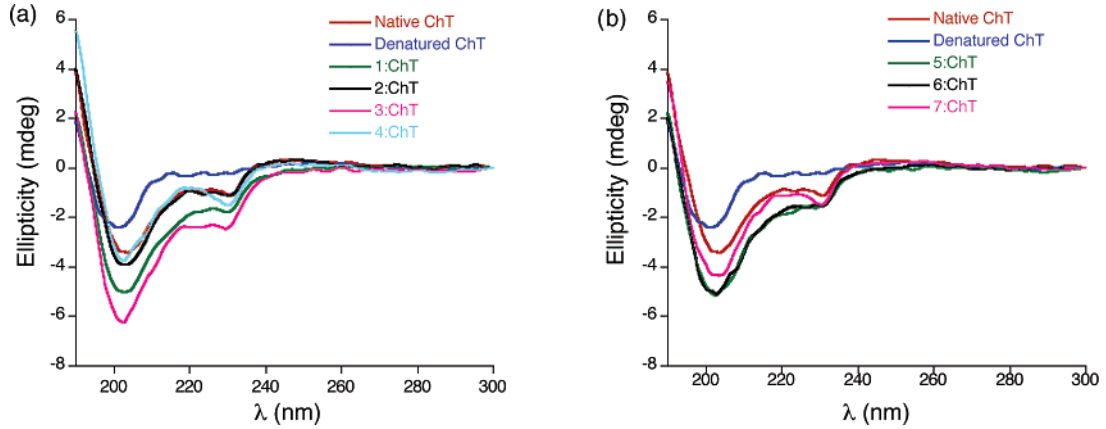


Figure 2. CD studies: (a) amphiphilic biaryl dendrimers, (b) classical amphiphilic dendrimers; 3.2 μM ChT incubated with 5.0 μM dendrons. The solutions were incubated in 5 mM sodium phosphate buffer pH 7.4 for 1 h. The spectra were compared with the ChT native and ChT thermo denature spectra.

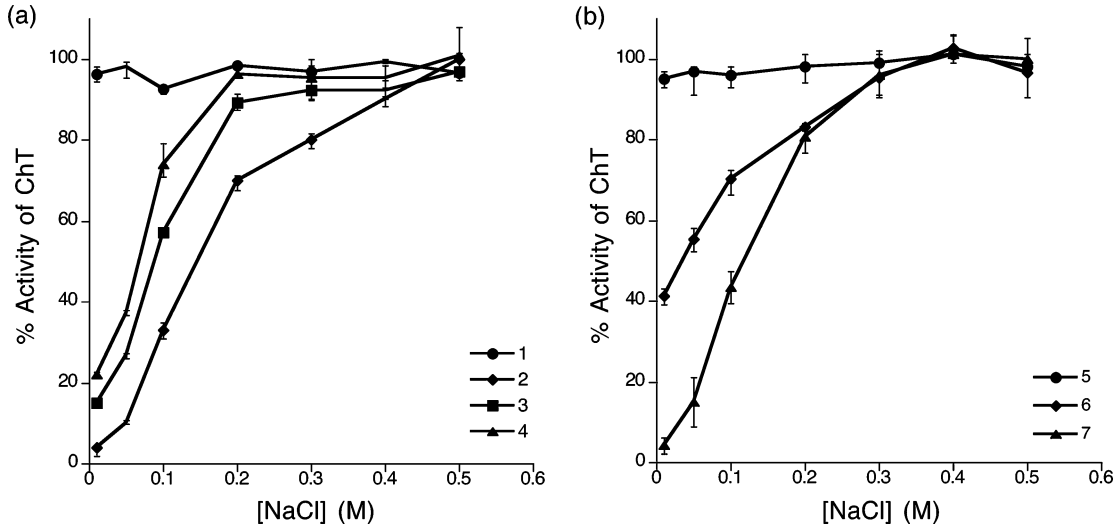


Figure 3. Effect of ionic strength: (a) amphiphilic biaryl dendrimers, (b) classical amphiphilic dendrimers; the ChT was incubated with dendrons in the presence of varied concentrations of NaCl. The data were normalized to account for the enhanced activity of ChT due to the salt effect.

events upon the structure of the protein. For example, we should investigate whether the observed inhibition is due to partial or complete denaturation of the proteins. To probe these possibilities, circular dichroism (CD) experiments were carried out. The CD spectrum of native ChT shows two characteristic peaks at 232 and 204 nm.[12] Thermal denaturation of ChT results in the loss of the peak at 232 nm, and a blue shift is observed for the peak at 204 nm, as could be seen in Figure 2. The far-UV CD spectra of all dendrimer/ChT complexes illustrated no significant change in the CD spectrum. These results suggest that the binding event does not result in denaturation of the protein.

Since the interaction is noncovalent, based on electrostatics, and since the binding event does not denature the protein, it should be possible to recover the enzymatic activity of the protein by screening the electrostatic interaction in solution. A common way of doing this is to increase the ionic strength of the solution.[10h,11] To investigate the possibility of the release of ChT from the dendrimer surface, enzymatic activities of dendrimer/ChT complexes were studied at different salt concentrations ranging from 0.01 to 0.5 M NaCl. Control experiments were conducted using the same concentration of ChT and NaCl without the dendrons for each data point. Each % activity of ChT in Figure 3a and 3b was normalized to this control experiment. The binding and inhibition effect of the ChT−dendron complex were strongly dependent upon ionic strength, as shown in Figure 3. The activity of ChT was dramatically recovered by increasing the salt concentration from 0.01 to 0.2 M. Thus, it is clear that the preliminary binding of the dendrimer to protein is based on electrostatic interaction and is reversible. The efficiency of binding lessened due to disruption of the electrostatic interaction by increasing the ionic strength in solution.

From the studies so far, it is clear that both classes of dendrimers interact with the protein through electrostatics and that this interaction is reversible. However, the binding affinity and binding ratio of this interaction are different for these two classes of dendrimers. These have been attributed to the fact that the biaryl dendrimers contain a carboxylate moiety in each repeating unit and that the biaryl dendrimers are preorganized into a micelle-type assembly. We were interested in finding out whether this difference affects the function of the dendrimer−protein complex. Also, the systematic differences in each generation of the dendron provide us an opportunity to gain certain insights into the nature of the interaction between ChT and multivalent ligand scaffolds in general. For example,

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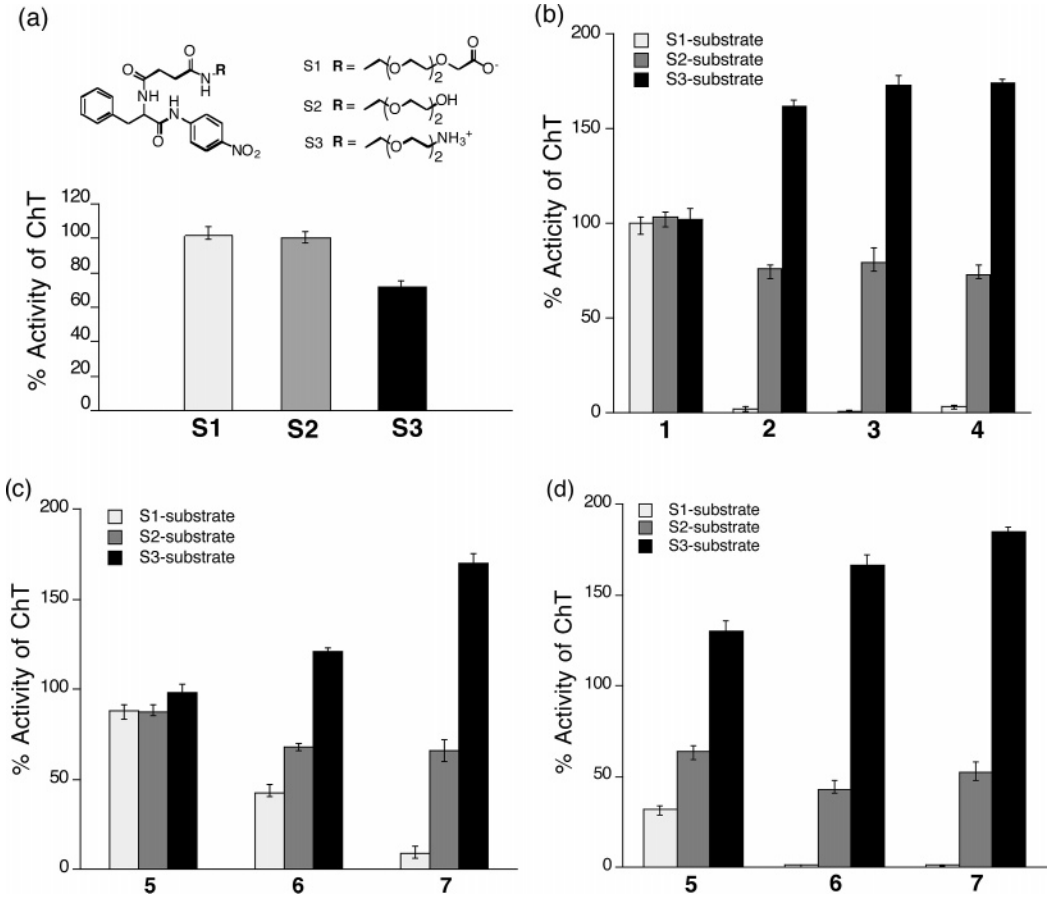

Figure 4. Substrate selectivity: (a) Activity of ChT (3.2 μM) with different substrates; (b and c) normalized activity of ChT (3.2 μM) with different substrates. Condition: 3.2 μM ChT was incubated in 75 μM **1**; other samples were incubated in 25 μM **2**, 10.7 μM **3**, 5 μM **4**, 37.5 μM **5**, 18.8 μM **6**, and 9.4 μM **7** (all dendrons have the identical number of COOH groups = 75 μM). (d) Normalized activity of ChT (3.2 μM) with 200 μM **5**. Other samples, ChT was incubated in 98 μM **6** and 47 μM **7**, respectively. All solutions were incubated in the presence of 5 mM sodium phosphate buffer pH 7.4 for 1 h.

recently, it has been shown that an electrostatically based complex of artificial molecular scaffolds with ChT results in an enzymatic activity that is dependent on the charge of the chromogenic substrate.[10f,11] In those studies, it has been shown that the negatively and positively charged substrates (**S1** and **S3**) exhibit significant inhibition and hyperactivity, respectively, in the presence of the negatively charged ligand scaffolds. While these results are attributed to the repulsive or attractive interaction between the substrate and the ligand, the inhibition observed with neutral substrates has been attributed to steric hindrance of the active site. Dendrons, under study here, provide a unique opportunity to test the latter part of the hypotheses. This is because the Fréchet-type dendrimers **5**−**7** represent systematic, small increments in size of the ligand scaffold, while our biaryl dendrimers **2**−**4** represent a rather large increase from **7** due to aggregation. To investigate this structure−property relationship, we carried out enzymatic assays with three different substrates **S1**−**S3**. Results of these studies are outlined in Figure 4.

First of all, it should be noted that, in order to understand the influence of the dendritic molecule upon the enzymatic activity, we carried out the necessary control experiments with each of the substrates under identical conditions, in the absence of the dendrons. The relative activities shown in Figure 4b−d are normalized to these controls. Nonetheless, the inherent relative activities of the native ChT toward each of the substrates **S1**−**S3** are shown in Figure 4a.

Figure 4b shows the activity of ChT against three substrates in the presence of compounds **1**−**4**. In this case, a 3.2 μM solution of ChT was incubated with 75 μM, 25 μM, 10.7 μM, and 5 μM solutions of **1**−**4**, respectively, Different concentrations of dendrons are taken here to ensure that the relative amounts of carboxylate units vs the number of protein molecules are similar. Activities of each of these dendron−ChT combinations were examined against each of the three substrates **S1**−**S3**. While compound **1** does not affect any change in the enzymatic activity, compounds **2**−**4** are able to exhibit a significant difference in reactivity toward the three substrates. This is understandable, because compound **1** exhibits no discernible binding toward the enzyme and therefore does not exert any difference in activity. On the other hand, compounds **2**−**4** exhibit large inhibition, minor inhibition, and hyperactivity against substrates **S1**−**S3**, respectively. However, it is interesting to find that there is no difference in magnitude of selectivity among the dendrons **2**−**4**. This is perhaps understandable, since the amount of aggregation in all these three dendrons is sufficient to exert the maximum effect on substrate selectivity.

On the other hand, since the size of the dendrons change more systematically with the Fréchet-type dendrons, we investigated the effect of the dendron generation on the substrate selectivity of the dendron/ChT complex. When maintaining the relative ratio of the carboxylates and the protein molecule the same as above, the results obtained are shown in Figure 4c. It is

intriguing to note that there seems to be a dendritic effect upon substrate selectivity. However, it is important that we rule out the alternate possibility, which involves the fact that there is a larger variation in the binding affinity of dendrons **5**−**7**. Therefore, the results in Figure 4c could be a simple manifestation of the bound vs unbound enzymatic activity. Indeed, when we increased the concentration of the dendrons to ensure that most of the proteins are in the bound form (using the binding affinities shown in Table 1), the substrate selectivities obtained for dendrons **6** and **7** were very similar to those for compounds **2**−**4** (Figure 4d). On the other hand, the substrate selectivity for the dendron **5** increases but does not reach the maximum due to the very low binding affinity of the dendron. Further increase of the concentration of **5**, to ensure that most of the proteins are bound, results in a precipitation from the buffer solution.

The above results show that the size of the ligand scaffold does not have a significant effect on the substrate selectivity of the enzyme. One could hypothesize that the selectivity is then driven mainly by electrostatics. It is also important to point out another intriguing result in the case of the enzymatic activity in the presence of dendrons **2**−**7**. As mentioned above, the inhibition of the enzymatic activity against the neutral substrate **S2** has been attributed mainly to the steric effect that the ligand scaffolds could provide for the substrate to access the active site. If this were the case, one would expect the relative activity of the enzyme to decrease with respect to **S2**, when moving from dendron **6** to **7**, since the latter dendron is bigger. However, this was not the case. If anything, there was a slight increase in the relative activity in the presence of **7**. Similarly, one would expect dendrons **2**−**4** compared to **6** and **7** to have much lower activity against neutral substrates due to the larger aggregate size. Once again, we found this not to be the case. Therefore, the argument that the activity against the neutral substrate is indicative of the steric crowding by the ligand scaffold does not seem to be correct. The binding likely causes an inherent change in the enzymatic activity, in which steric crowding could be a minor contributor. But, there is also some other factor involved; an allosteric change in the enzyme that is not discernible by absorption or CD spectroscopy is an example of a possibility. However, since we do not have any spectroscopic evidence for such an effect, this proposed possibility remains speculative at this time.

Next, to identify whether unbound carboxylates present in the dendrimers could be the reason for recruiting the substrates closer to the active site and therefore an increase in activity for substrate **S3**, we varied the relative concentration of the dendrimer vs ChT (all concentrations above the binding affinity and CMC). The idea is that this increase will afford an increased number of free (unbound) carboxylates in dendrimers. We found that the selectivity is independent of the concentration of the dendrimer. This is similar to what we observed with our amphiphilic polymers as well. These results suggest that the unbound carboxylate is not a primary source of the observed selectivity.

Summary

We have used biomolecular recognition as the probe to investigate the properties of the recently reported facially amphiphilic biaryl dendrimers by comparing these molecules with the corresponding amphiphilic benzyl ether dendrons. From these systematic studies, we show that: (*i*) the carboxylic acid functionalities present in the internal layers as well as the periphery are available for molecular recognition in the biaryl dendrons. (*ii*) Preorganization of multiple copies of ligands through aggregation in the biaryl dendrimers results in high binding affinities, even for small dendrons. (*iii*) A G-3 dendron containing eight copies of the ligands in a classical, benzyl ether dendron is the critical size to approach the binding affinities exhibited by the large preorganized particles for ChT. (*iv*) The systematic variations in the dendron sizes also allowed us to show that attributing the observed inhibition with neutral substrate **S2** to steric effect is not correct. Gaining fundamental insights regarding the relative recognition capabilities of the facially amphiphilic biaryl dendrons will open up new possibilities for these amphiphilic assemblies in applications such as targeted drug delivery.

Experimental Section

α-Chymotrypsin (ChT) from bovine pancreas (E.C. 3.4.21.1) and all other chemicals were purchased from Sigma Aldrich chemical company. Dendrons **1**−**4** were synthesized using our previously published procedure.[5a] Dendrons **5**−**7** were synthesized by following literature protocols.[8a,9]

Enzymatic Activity Assay: Enzymatic hydrolysis was monitored using a microplate reader (EL808IU, Bio-Tek Instruments, Winoosk, VT). The reactions were carried out in 5 mM sodium phosphate buffer pH 7.4 with 3.2 μM ChT in either a varied concentration of dendrons or specified. The enzymatic hydrolysis was taken by adding 16 μL of 26 mM substrate to 184 μL of preincubated ChT−dendron supernatant. Hydrolysis of substrate was monitored for 10−30 min at 405 nm. The assays were recorded in triplicate, and the average values were reported with less than 10% standard deviation.

Circular Dichroism (CD): Far-UV CD spectra were obtained from 190 to 240 nm in a Jasco J-720 (Jasco Instruments, Tokyo, Japan) spectrophotometer with a 1 mm path length quartz cuvette. ChT (3.2 μM) was incubated with dendrons (5 μM) in 5 mM sodium phosphate pH 7.4 for 1 h. Three scans were averaged at a rate of 20 nm min^{-1} with a sample interval of 0.2 nm and an 8 s response. The temperature was fixed at 25 °C.

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Supporting Information Available: Syntheses and procedure for the estimation of binding constants. This material is available free of charge via the Internet at http://pubs.acs.org.

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