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Differentiation of Functional Groups and Biologically Relevant Anions Using AT-PAMAM Dendrimers

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Abstract

Biological anions have often proven to be difficult analytes to differentiate in solution. Many of the anions bear similar structural characteristics and similar charge states. Using a commercially available indicator and AT-PAMAM dendrimers, a sensing ensemble for a number of biological anions was constructed. The system was able to identify each of the different analytes as well as the regioisomers of three different tricarboxylates. The system shows responsiveness to not only functional group identity but also the charge state and identity of the anionic species.

Keywords

PAMAM dendrimers; anion detection; pattern recognition; functional group differentiation; phosphate

Introduction

Many biological processes rely on anionic phosphate or carboxylate based cofactors in order to carry out their desired biological function. Some cofactors, such as NAD and NADP, serve as oxidation/reduction reagents, whereas others act as electron donors, or as energy currency for key biological processes such as the generation of ATP and the degradation of cell nutrients.¹ Given the importance of cofactors to biological homeostasis, monitoring their levels as well as their regulation is important for studying and understanding many disease states.

Numerous other groups have studied anion sensing with particular focus on small ions or high specificity for a particular anionic class.² Particular emphasis has been placed on phosphate due to its biological relevance.³ For example, the Gunnlaugsson group has developed several anion receptors based upon a urea-amide linkage which is selective for phosphates.^{2a, b} Others, such as the Sessler, Saha, Beer, Steed, Gale and Lippard groups, have developed selective anion receptors for particular ions such as fluoride^{2l, 4}, carboxylate^{2e, 2k}, and chloride^{2m, 5} as well as phosphate^{2n, 6}.

While these systems have a particular ion as a target, several sensor systems have been developed that use multiple receptors that can pattern different types of anions or particular mixtures of anions. Work from the Anzenbacher group has used an array of calix[4]pyrroles to identify anions, particularly fluoride ions, where the system was used to identify this anion, as well as its relative concentration in several toothpaste brands.²ⁱ

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Among the most studied anions are the nucleotide triphosphates (NTP's), for which numerous methods of detection have been developed, including some by our own group.⁷ We previously showed that a trisaminobenzene scaffold with multiple peptidic arms, which impart differentiation and some enhanced binding, can be used to differentiate ATP from GTP and AMP in aqueous media using multivariant data analysis, such as PCA.⁸

However, only a handful of supramolecular methods have been developed for the detection of NAD or NADP and their reduced counterparts. In 1997 and 1998, Lehn and then Bianchi reported receptors for NAD and NADP based upon a polyammonium macrocycle.⁹ Lehn added an acridine group to bind the nucleotide through π -stacking interactions, and he reported binding constants of approximately 10^7 to 10^8 M⁻¹ in water. Bianchi used a protonated azacrown ether to bind the anionic phosphate group, and explored the redox potential of the nicotinamide to monitor NAD levels.^{9b} In addition, the Hong group developed a zinc coordinated dipicolylamine receptor that bound FAD, another biological anion of interest.¹⁰ By using the natural fluorescence of FAD, they were able to identify the binding of FAD over PPi.

In recent years, our group, along with others, have used arrays of small molecule receptors that allow for the differentiation of similar analytes in complex media.¹¹ Using arrays of receptors with chemometric techniques to analyze the data, our group has been able to successfully identify analytes in solution¹² as well as identify complex mixtures.¹³

Additionally, our group has studied the use of large macromolecular structures such as PAMAM dendrimers and bovine serum albumin (BSA) as scaffolds for the development of supramolecular receptors.¹² Recently, we published a study of the uptake of negatively charged indicators by polycationic dendrimers.¹³ The stoichiometry and structures of the dendrimer complexes revealed multi-component assemblies, as indicated by UV/Vis spectroscopy, fluorescence, fluorescence anisotropy, and isothermal titration calorimetry (ITC).¹⁴

In this study, we report the use of four generations of amino-terminated PAMAM dendrimers with the fluorescent reporter, 5(6)-carboxyfluorescein (CF), to differentiate biologically relevant anions and other polyanions in aqueous media. PAMAM dendrimers are large polymeric like structure which radiate in multiple directions and have a finite number of repeating units.

In the case of PAMAM dendrimers (structure of G2 shown above), those repeating units are ethylenediamine and propionic acid resulting in a large number of terminal functional groups that increase as a factor of 2 per generation. Dendrimers, unlike traditional polymers, have a large degree of uniformity, size, and large degree of terminal functionalization. Using UV/vis, fluorescence, and fluorescence anisotropy measurements, coupled with chemometrics, we differentiate nine biologically relevant polyanions. Furthermore, the dendrimer assemblies were used to differentiate three different regioisomers of benzene tricarboxylate.

Experimental Methods

G3-G7 PAMAM dendrimers (5–20% MeOH solutions) and 5(6)-carboxyfluorescein were purchased from Sigma-Aldrich and used as received. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Fisher Scientific and used without further purification. All biological anions were purchased from Chem-Impex International except for folic acid, which was purchased from Sigma Aldrich. 1,3,5-Benzenetricarboxylic acid was purchased from Sigma Aldrich while 1,2,3-benzenetricarboxylic acid and 1,3,4-benzenetricarboxylic acid were purchased from AK Scientific. A 50 mM HEPES buffer was prepared using millipore water from a Siemens purification system and the pH was adjusted to 7.4 using aqueous solutions of NaOH and HCl and these solutions were filtered and degassed via sonication. All experiments were carried out in this buffer.

Each spectroscopic reading was carried out using a BioTek Synergy 2 Multimode Microplate reader. 96-Well black plates on an optically clear plastic bottom (NUNC plate #265301) were used to allow for simultaneous UV/vis and fluorescence detection on the instrument. Each plate contained a 2 μ M CF solution which was used to reference the instrument and to minimize plate to plate reproducibility. The plates were referenced to the 2 μ M CF, which was the highest concentration of CF, making these wells the maximum intensity recorded in each series. Each well was repeated in at least triplicate to reduce random errors.

Before studying the interaction of anions, the PAMAM dendrimers were titrated into a constant concentration of the **CF** indicator. In our previous work, a maximum loading value of **CF** to the dendrimer had been obtained. The inflection point obtained where the addition of more dendrimer to **CF** no longer caused a signal modulation in the fluorescence intensity, or in the fluorescence anisotropy, were used for all anion displacement and patterning studies.

Using a set ratio of dendrimer with **CF**, shown in Table 1, different anions were titrated into these solutions and the resulting signal modulations of the **CF** were recorded. The introduction of anions caused the fluorescence intensity of **CF** to increase and the fluorescence anisotropy to decrease.

In the patterning studies, a set amount of each anion (50 μM) was placed in a well with a fixed ratio of dendrimer and **CF** indicator based upon the fluorescence intensity ratio in Table 1 with a total volume of solution of 300 μL . Each generation of dendrimer was used in its own plate to prevent plate-to-plate variations from being detected when conducting the chemometric differentiation of the anions. All chemometrics were conducted in Microsoft Excel 2003 with Alladinsoft's XLSTAT 2009 software.

Results and Discussions

While polycationic PAMAM dendrimers can be used for the sequestration of anionic structures, there is no direct method by which an optical signal can be modulated upon the binding of target anions because the dendrimer does not contain a chromophore. While it has been shown that fluorescein can be bound covalently to the terminal amines, the modification of the dendrimers is a complicated process.¹⁵ Furthermore, the modification prevents the use of anisotropy as a signaling modulation for the detection of anions in solution.

Given our previous reports that dendrimers sequester a large number of anionic indicators,^{13–14} with particular emphasis on the anionic **CF** indicator, we felt a signaling methodology could be developed. Based upon this previously reported phenomena, we postulated that **CF** and dendrimer shows potential for use in an indicator displacement assay. An indicator displacement assay requires that the **CF** show a different optical signal between the bound **CF** and the **CF** that is displaced into the bulk solution by the target anion. This equilibrium is described in Scheme 1. In order to probe this signaling methodology, we sought to use two small commercially available polyanions to probe the ability of the displacement of **CF** from bound dendrimer as a signal.

In our first study, we used tri- and tetracarboxylates to probe the displacement of **CF** from the dendrimer. The target anions, trimesic and pyromellitic acid, were used because they have low UV/Vis absorbance, no fluorescence, and are commercially available. To test the displacement, we set the ratio of dendrimer (**D**) to **CF** at a point that the anisotropy was at 90 percent of its maximum (G4 2.5 **D** : **CF**, G5 1.9 **D** : **CF**, G6 2.1 **D** : **CF**, G7 1.6 **D** : **CF**). At these ratios, the dendrimer contained only 1 to 2 **CF**, and thus the assemblies contained an excess of positive charge to be paired with the anions. Figure 1 shows a titration of trimesic acid into this fixed ratio for generations of dendrimer and the **CF** indicator. As can be seen in the figure, the titration of the anion into the solution caused an increase in the fluorescence intensity, with an accompanying decrease in anisotropy toward the baseline value. These curves show a sigmoidal shape like due to the sequestration of anions before the displacement of the indicator from the dendrimer. This phenomenon was extensively explored previously.¹⁴ A similar titration was also carried out with pyromellitic acid, 1,2,4,5-benzenetetracarboxylate, with the same ratio of **CF** indicator to dendrimer as with trimesic

acid. The pyromellic acid showed restoration of the fluorescence intensity at lower equivalents than trimesic acid with the trimesic acid showing a gradual increase in fluorescence intensity while the pyromellic acid showed a more pronounced sigmoidal shape. In addition to the restoration of fluorescence intensity, the fluorescence anisotropy upon addition of pyromellic acid also decreased in all generations to the anisotropy of free **CF**, unlike trimesic acid in which only G3 and G4 returned to the anisotropy of free **CF** indicator, even at 800 μM **CF**. These changes indicate that the tetraanionic pyromellate shows a higher binding affinity than trimesate for the polycationic dendrimer. While these carboxylates served as a useful proof of principle that target anions can generate a measurable optical signal, the dendrimer to **CF** indicator ratios proved less than optimal because greater than 100 μM of the anions are needed to modulate the optical signal.

In order to improve the sensitivity of the assay, we changed the ratio of **CF** to dendrimer. Instead of using of ratio of dendrimer to **CF** at which the anisotropy signal was 90 % saturated, we choose the point in which the fluorescence intensity stopped significantly decreasing when titrating dendrimer into **CF** (see Fluorescence Intensity in Table 1 for ratios). At this point, the **CF** indicator is no longer being taken up by the dendrimer, and thus we postulated that the addition of an anion would most effectively cause displacement of the indicator, and consequently restore the fluorescence while decreasing the anisotropy of the **CF** indicator. Hence, we expected that a smaller concentration of the target analyte would cause a signal modulation, and therefore the assay would be more sensitive to small changes in anion concentration.

In order to explore the displacement at this lower indicator to dendrimer ratio, we choose to explore three different anions: ATP and NADP, both that contain anionic phosphates, and trimesic acid that contains carboxylates. The phosphate group is prevalent in many common cofactors, making the anionic group an interesting target for study and differentiation. Many of the phosphate-containing cofactors have very similar structures making them difficult to differentiate using supramolecular techniques.

We first studied 1,3,5-benzenetricarboxylate, and followed its ability to displace **CF** from the dendrimers. For all generations, we saw a similar pattern of displacement as with the higher ratio of dendrimer to **CF** used in the initial studies of trimesic and pyromellic acids. The extent of displacement, however, was higher at significantly lower concentrations of anions compared to these early studies. The displacement at lower concentrations demonstrated that this lower ratio of **CF** indicator to dendrimer creates a more sensitive assay. As can be seen in Figure 3, both the fluorescence intensity and the fluorescence anisotropy can be monitored to reveal the extent of binding between the dendrimer and the target anionic analyte.

While either the fluorescence intensity or fluorescence anisotropy spectra could be used separately in our efforts to differentiate biologically relevant anions, the plots support the idea that using both spectra can provide additional useful information in the discrimination of the target anions. For example, the intensity plots show a return of fluorescence, while the polarization plots do not follow a simple increase, and each generation does not start at the same value, thereby leading to differing changes in fluorescence polarization. This data reveals that both emission and polarization should be monitored to enhance the potential for differentiation.

Before using the optical data for anion patterning, we decided to test the differences other anions would show when displacing **CF**. Hence, ATP and NADP were targeted because these two analytes contain multiple anionic groups but also contain the same head groups that might hamper differentiation.

Upon addition of ATP to **CF** loaded dendrimers, displacement of the indicator was observed similar to that with the tricarboxylate, yet the displacement occurred at lower anion concentration. The increased displacement of **CF** by ATP is likely due to increased charge density of the phosphate tail of ATP relative to the benzene tricarboxylates. While the charge density is higher, the triphosphate may also form a better array of hydrogen bonding and salt bridge partners with the amines of the dendrimers that further facilitates displacement. Additionally, the polarization data showed some interesting trends in the larger generations. While G3 and G4 showed simple displacement curves, the larger generations G5–G7 showed an increasingly sigmoidal displacement curve, as well as starting at a significantly higher initial polarization (Figure 4). These differences in the fluorescence intensity and fluorescence polarization between the anions, and the generations, were expected to further improve the ultimate chemometric results for differentiating the multiple anions. These studies proved that other anions could displace the dendrimer bound **CF** to differing extents, giving evidence that the target anions could be patterned in water.

In order to pattern the targeted anions, each would need to have different binding affinities with the different generations, and thus we studied the binding curves for NADP to test if this anion gave a different profile than ATP. As can be seen in Figure 5, the binding isotherms for NADP, both fluorescence intensity and the fluorescence polarization, are clearly different from those of ATP (Figure 4). The isotherms show that significantly more NADP is needed to lead to the same level of indicator displacement as when ATP was used. In addition, there is a much more pronounced sigmoidal shape for the larger generations of dendrimers for both the intensity and polarization data for the ATP. The differences are accentuated when the isotherms are overlaid (Figure 6). This provides evidence that chemometrics could differentiate the target anions. Several other anions were titrated into the same fixed ratio of **CF** and dendrimers and gave different displacement isotherms.

The patterning studies were carried out with five generations of dendrimers. Each generation was used in its own individual plate to prevent misclassification based upon generation rather than anion identity. Each plate contained the same ratio of **CF** indicator and dendrimer along with a fixed amount of each anion. Each plate was analyzed on a 96 well plate reader giving a fluorescence intensity and polarization reading, as well as a UV/Vis spectra from 460–510 nm. The fluorescence intensity, polarization, and UV/Vis absorbance at 495 nm were entered into XLStat and used to produce a linear discriminate analysis (LDA) plot (Figure 7). As can be seen, each of the anions is separated, but some of the anions we identified as having weak interactions with the dendrimers cluster very close together. Yet, the jack knife analysis showed a 100 % correct classification of each anion. Furthermore, the FAD anion was clearly differentiated, but that is due to the increased absorbance of the FAD anion in the same region as the **CF** indicator. An additional LDA plot (Figure 8) was generated which excluded the weak binders and FAD to allow for the maximum amount of differentiation of those anions.

The F1 axis, which shows the largest portion of the differentiation, is directly related to the binding ability of the anion to the cationic dendrimers. While there is some correlation with the amount of charge on each anion, as can be seen in Table 2, the total amount of charge is not directly related to the location of the anion on the F1 axis. As such, the regiochemistry of the anionic charges is important in the patterning. Yet the different generations of dendrimers themselves do not possess much cross reactive ability as they all primarily respond to the amount of charge on the dendrimer. The larger generations (G5–G7), however, have different reactivity than the smaller generations of dendrimers (G3–G4). The smaller generation dendrimers tend to respond more linearly to anions with more charge density while the larger generations tend to have a more sigmoidal based upon the amount of

charge on the anion. These small differences between generations most likely account for the differentiation along the F2 axis.

As stated, the differentiation of the carboxylate derivatives was not dependent upon their charge alone. For example, we analyzed the three possible regioisomers of a tricarboxylatebenzene: a 1,3,5; a 1,2,3; and a 1,2,4 derivative. Each of these isomers contain the same number of charges making them an interesting set of structures for determining if the dendrimers can differentiate the anions based upon their regiochemistry. As can be seen in the Figure 9, each of these regioisomers have been clearly discriminated from each other indicating that the location and order of anion placement is important to binding. The location of the anionic sites in the tricarboxylates is anticipated to influence their salt bridges with the dendrimers leading to differential displacement of the **CF** indicator. Based upon the raw data, we believe that the all meta arrangement of carboxylates, 1,3,5 regioisomer, forms the optimal salt bridges with the amines on the dendrimer.

Conclusions

In this paper we have reported a differential sensing scheme using a commercially available indicator and PAMAM dendrimers, that gives classification of several biologically relevant anions in water at physiological pH. In addition to the biological anions, the sensor suite is able to differentiate the regioisomers of an organic trianion. The system has sensitivity for anions at low μM level in water, with full differentiation of all the anions studied. The differentiation arises primarily from charge density, but also regiochemistry and functional group identity.

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Figure 1. Isotherms for the displacement of 5(6)-carboxyfluorescein from a [D-CF] complex by the trimesate trianion. Top: fluorescence intensity; Bottom: fluorescence anisotropy.

Figure 2. Isotherms for the displacement of 5(6)-carboxyfluorescein from a [D-CF] complex by the pyromellitate tetraanion. Top: fluorescence intensity; Bottom: fluorescence anisotropy.

Figure 3.

Isotherms for the displacement of 5(6)-carboxyfluorescein from a [D·CF] complex by the trimesic trianion at low dendrimer to CF ratio. Top: fluorescence intensity; Bottom: fluorescence anisotropy.

Figure 4. Isotherms for the displacement of 5(6)-carboxyfluorescein from a [D-CF] complex by the ATP tetraanion at low CF to dendrimer ratio. Top: fluorescence intensity; Bottom: fluorescence anisotropy.

Figure 5. Isotherms for the displacement of 5(6)-carboxyfluorescein from a $[\mathbf{D}\cdot\mathbf{CF}]$ complex by the NADP tetraanion at low CF to dendrimer ratio. Top: fluorescence intensity; Bottom: fluorescence anisotropy.

Figure 6.

Isotherms for the displacement of 5(6)-carboxyfluorescein from a [D-CF] complex by the ATP tetraanion at low CF to dendrimer ratio overlaid with the displacement isotherms for NADP tetraanion. Top: fluorescence intensity; Bottom: fluorescence anisotropy.

Figure 7.
LDA plot of all biological anions of interest (Jack knife Analysis = 100%)

Figure 8.
LDA plot of all biological anions of interest to which displacement of CF could be detected to an appreciable extent

Figure 9.
LDA plot of all tricarboxylate anions

Scheme 1.

The indicator displacement assay (IDA). D = dendrimer, A = anion, CF = 5(6)-carboxyfluorescein

Table 1

Ratio of CF to dendrimer for each generation and each methodology upon which signal first appeared to saturate

Generation	Ratio (Anisotropy)	Ratio (Fluorescence Intensity)
G3-NH2	N/A	1 CF : 1 D
G4-NH2	2.5 CF : 1 D	2 CF : 1 D
G5-NH2	1.9 CF : 1 D	3 CF : 1 D
G6-NH2	2.1 CF : 1 D	5 CF : 1 D
G7-NH2	1.6 CF : 1 D	10 CF : 1 D

Table 2

Anions with total charge versus position on the F1 axis of the LDA chart

Anion	Net Charge	Location on LDA (L to R)
Flavin Adenine Dinucleotide	2	1
Adenosine Monophosphate	2	2
Thiamine Pyrophosphate	2	3
Nicotinamide Adenine Dinucleotide, Oxidized	1	4
Folic Acid	2	5
Nicotinamide Adenine Dinucleotide, Reduced	2	6
1,2,3-Benzenetricarboxylate	3	7
Adenosine Diphosphate	3	8
1,3,4-Benzenetricarboxylate	3	9
1,3,5-Benzenetricarboxylate	3	10
Nicotinamide Adenine Dinucleotide Phosphate, Oxidized	3	11
Nicotinamide Adenine Dinucleotide Phosphate, Reduced	4	12
Adenosine Triphosphate	4	13