J|A|C|S COMMUNICATIONS

Published on Web 10/29/2009

Characterization of Protein Aggregation via Intrinsic Fluorescence Lifetime

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Aggregation plays an integral role in many cellular pathways, one of the most important being mediation of the infection and proliferation potential of tumors and pathogens.[1] Protein aggregation has also been implicated in pathological conditions such as Alzheimer's and other amyloid-related diseases.[2] Because of the importance of multivalently displayed carbohydrates on cell surfaces, sugar-induced aggregation has drawn considerable attention.[3,4] Sensor strategies based on controlled aggregation have been reported for the detection of toxins and other biologically relevant compounds.[5] Multivalent interactions often involve multiple weak monovalent binding events. An in-depth understanding of aggregation in complex systems requires studies that go beyond measuring the monovalent association constants. Particularly valuable would be methods capable of characterizing aggregation events in real time.

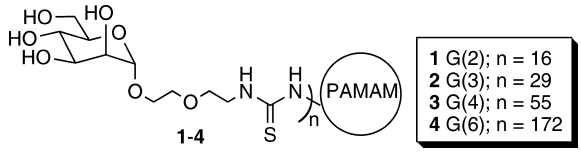

Figure 1. Mannose-functionalized polyamidoamine (PAMAM) dendrimers **1**−**4**.

Here we present important new information about the aggregation of the mannose-specific lectin Concanavalin A (Con A) by glycodendrimers **1**−**4** (Figure 1, prepared as described in ref 6). Glycodendrimers are very well suited for studying the formation and mediation of multivalent interactions. We have reported in several previous studies on the Con A−glycodendrimer system that binding and inhibition efficacies depend on size and functionalization.[6] In this case, we applied Con A intrinsic fluorescence in a novel time-domain format.

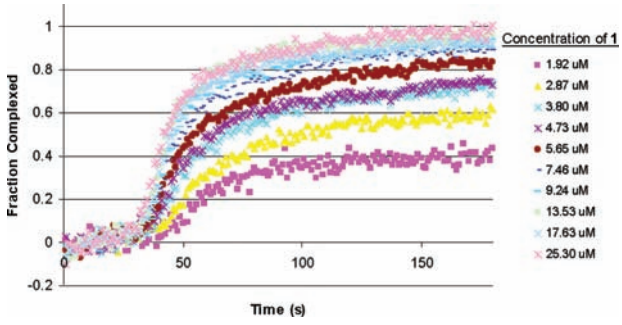

Figure 2. Complex formation upon addition of **1** to Con A (100 ug/mL).

Intrinsic fluorescence is attractive because of its label-free aspects, but light scattering and inner filter effects associated with the extensive precipitation that often accompanies aggregation hamper steady-state approaches. Fluorescence lifetime approaches are often stated to be immune to precipitation problems because the desired information can be extracted from the shape of the decay curve rather than the intensity.[7,8] However, conventional lifetime technology is too slow for our needs, as we have been studying reactions with half-lives as short as just a few seconds. The data reported here were collected with a prototype instrument that increases by a factor of ∼100 the rate at which fluorescence lifetime data can be collected (see the Supporting Information for details).

The experimental configuration is straightforward. Known amounts of glycodendrimer solution were added to 2000 μL of 100 μg/mL Con A in a well-stirred cuvette at 25 °C. The baseline of Con A fluorescence (λ_{ex} = 295 nm, λ_{em} = 335 nm, 5 nm bandpass) was established for 30 s before the glycodendrimer aliquot was added. Fluorescence decay waveforms were measured once per second for the next 130 to 220 s.

The term "fluorescence lifetime" is used for convenience in this communication. However, our method does not follow the usual practice of reducing each raw decay curve ("waveform") to a set of lifetimes and amplitude factors via iterative reconvolution. As explained in the Supporting Information, we instead fit the waveforms as a linear combination of free and complexed Con A basis waveforms (Figure 2 and Figure S3). In essence, the fluorescence decay waveforms are treated as spectra in which the independent variable is time after excitation instead of wavelength. It should also be noted that complex formation and precipitation have minimal effects on the wavelength spectral distribution of the Con A fluorescence.

Control experiments showed that the primary sugar−Con A binding events are not the source of the observed fluorescence changes. Several millimolar concentrations of α-*O*-methyl mannoside (Me-man) (Figures S4 and S10) had minimal effect on the Con A fluorescence and did not result in precipitate formation. To ensure that the dendrimer framework itself was not the source of the Con A quenching, a galactose-functionalized dendrimer that does not bind to Con A was added under the same conditions as the mannose-functionalized dendrimers **1**−**4**. Again, no precipitation was observed visually, and nonspecific binding was minimal relative to baseline drift and noise (Figures S5 and S10).

The fluorescence changes are most likely associated with a Con A−Con A protein−protein interaction orchestrated by binding to the glycodendrimer framework. Figure 3 illustrates our hypothesis that a reversible protein−protein interaction between proximal Con A lectins causes the quenching. The primary role of the dendrimers is to hold the Con A lectins in close proximity, thereby increasing their effective concentration. A protein−protein interaction, presumably a conformational change affecting the environment of one or more Con A tryptophans, can then occur.

All generations of glycodendrimer showed saturation behavior, i.e., the fluorescence changes reached a plateau at sufficiently high

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10.1021/ja904073p CCC: $40.75

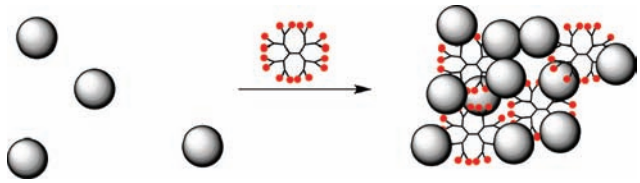

Figure 3. Glycodendrimer-mediated lectin aggregation: (left) uncomplexed Con A; (right) cross-linked state.

dendrimer concentration. Furthermore, the fluorescence decay curves at saturation were identical within the experimental uncertainty for all dendrimer generations. Thus, all of the fluorescence decay curves for the entire range of dendrimer concentrations and all generations could be fit to a linear combination of just two waveforms, corresponding to "free" and "complexed" Con A. We conclude that the environment of the complexed Con A is apparently not affected by the generation of dendrimer (Figure S16).

However, the rate of complex formation is sensitive to the dendrimer generation and concentration. The kinetics traces (Figures S14 and S15) often appeared biphasic, with the much faster first step generally accounting for >90% of the total change. Except for very low dendrimer concentrations, a first-order kinetics model fit the data very well for the first 70 s after dendrimer addition and yielded an apparent rate constant k_{obs} (Figure 4 and Figures S14 and S15).

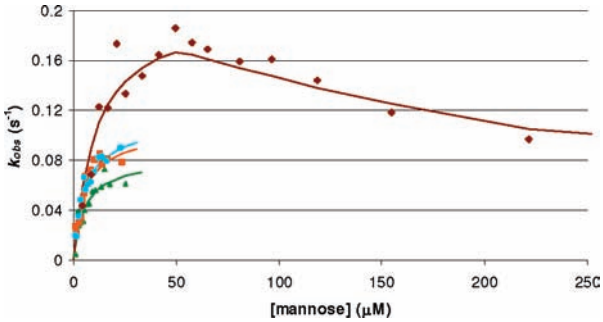

Figure 4. Representative k_{obs} values for Con A complex formation: **1** (▲), **2** (■), **3** (●), **4** (◆).

Although the mechanism is undoubtedly much more complicated than a simple first-order process, k_{obs} is a convenient way to depict how differences in dendrimer generation affect glycodendrimer-mediated protein aggregation. Comparing the results across dendrimer generations revealed an increase in the apparent rate constant with increasing dendrimer generation expressed on a per-mannose basis. At low concentrations of added dendrimer, there is a fixed pool of Con A lectins competing for a small number of glycodendrimers. The rate-limiting step in achieving a complex that changes the fluorescence decay waveform may be two Con A lectins becoming cross-linked into close proximity by a small number of glycodendrimers. Thus, the overall apparent rate constant increases as the concentration of glycodendrimer increases. As expected, the rate constant approaches a saturation value at sufficiently high dendrimer concentration, at which the rate-limiting step becomes the Con A−Con A mutual conformational change.

The multivalent binding in **4** boosts the kinetics over what is observed for the lower-generation dendrimers because it also serves to increase the Con A residence time on a sugar binding site. However, we see a slowing of the kinetics for very high dendrimer concentration in G6. The slower kinetics at higher G6 concentration can be understood as a consequence of some fraction of the Con A lectins initially being too far apart to undergo the protein−protein interaction (each Con A has more sugar binding sites to choose from) and the multivalent binding impeding their ability to move to new sites.

Because the same final degree of complex formation is achieved, we suggest that there is a slower process by which the Con A's migrate into adjacent positions on the dendrimer framework, which is the most stable configuration. Brewer and co-workers[9] have proposed a model in which lectin molecules bind and jump from carbohydrate epitope to epitope. We are proposing an attractive interaction between Con A molecules on adjacent glycodendrimer binding sites that causes their residence times to increase substantially. Notably, once the entropic penalty of Con A−sugar binding has been paid, only a small enthalpic stabilizing force is necessary for the protein−protein interaction. It is also interesting to speculate on a possible connection between our observations and the selectivity switching reported by the Whitesides group.[10]

In summary, fast and precise fluorescence lifetime experiments were performed using unlabeled lectin to characterize glycodendrimer-mediated protein aggregation. Lifetime measurements were used in these experiments to explain self-quenching phenomena induced by aggregation states, but this method is not limited to such and is viable for numerous binding studies. For example, intrinsic fluorescence can be used for the study of protein−protein interactions, protein−small-molecule interactions, vesicle and micelle formation, oligomerization events, and protein folding. Labeled compounds can be studied as well using the methods described here.

Acknowledgment. Support from NIGMS (R01 62444) and the Montana Board of Research and Commercialization Technology (Grant 08-48) is gratefully acknowledged.

Supporting Information Available: Methods, description of instrumentation, and fluorescence data for methyl mannose, galactose-functionalized dendrimers, and **1**−**4** with Con A. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA904073P