

Revealing dye and dye-drug aggregation into nano-entities using NMR

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ABSTRACT

It is becoming increasingly apparent that small molecules can self-assemble into a wide-range of nano-entities in solution that have intriguing properties. The recently introduced NMR aggregation assay is playing an important role in revealing these nano-entities. Here, we employ the NMR aggregation assay to expose the self-aggregation tendencies of dyes in solution. This dilution-based assay demonstrates that some dyes can exist as single-molecule entities whereas others can adopt aggregates of distinct sizes. Interestingly, dyes with highly related chemical structures can adopt largely different sized aggregates - demonstrating the existence of structure-nanoentity relationships (SNR) – which suggests that they can assume and/or be designed to have distinct properties. One property was evaluated where the drug Quetiapine (Seroquel) was added to the dye Congo red which resulted in the absorption of the drug into the dye nano-entity. This showed a direct drug-dye interaction, and it demonstrated that dye aggregates can have influences on drug solution behaviors. The NMR method described in this study provides a practical and valuable tool to monitor dye aggregates and to better understand their associated properties (e.g. toxicity, off-target activity) and potential utility (e.g. drug encapsulation, drug delivery systems).

1. Introduction

Small molecules can assume a wide range of behaviors in solution that can be considered within the context of a tri-phasic equilibrium [1,2]. That is, when a compound is placed in aqueous solution it can equilibrate between at least three states (Fig. 1). Some of the molecules can exist as soluble, fast-tumbling lone molecules that are completely diffuse, whereas others can form solid precipitate(s), and others can self-associate and adopt intermediate soluble aggregates or nano-entities. Notably, each compound likely has its own unique equilibrium signature and relative population among these states, and there is a critical dependence on many other factors such as buffer, co-solutes, etc.

The detection and quantification of a compound's signature equilibrium remains elusive to this day [2]. Whereas the solid precipitate phase is detected visually (or via a microscope for fibrils), the distinctions between the soluble lone-molecules and aggregate phases are not apparent. This is in part due to the limited detection methods available such as dynamic light scattering (DLS) [3]. DLS is a practical technique that can reveal the existence of large, micelle-like aggregates that are homogeneous. However, we recently introduced an NMR aggregation

assay that exposed a wide range of nano-entity sizes that can exist [1,2] especially small multimers, which are often undetectable by DLS. Fig. 1 shows how NMR is sensitive to compound tumbling rates and behavior for all three phases. That is, fast tumbling molecules exhibit sharp resonances (Fig. 1A), and solid precipitates result in extremely broad and unobservable resonances by solution NMR (Fig. 1D). Aggregates give rise to intermediate resonance attributes such as broad resonances and/or unusual features such as those shown in Fig. 1B and C. Thus, we chose to employ NMR in this study to explore the behavior of dyes in solution, which could then be a valuable tool to begin correlating with their properties. Here, we demonstrate that the NMR aggregation assay is a feasible method to explore the behavior of dyes in solution, which can then be a practical tool for correlating nano-entity properties.

2. Materials and methods

2.1. Compounds - dyes

The dyes and drug used in this study were all obtained from commercial vendors. The dyes and their CAS numbers are as follows: Azo Rubine (3567-69-9), Acid Blue 9 (3844-45-9), Erythrosin B (16423-68-

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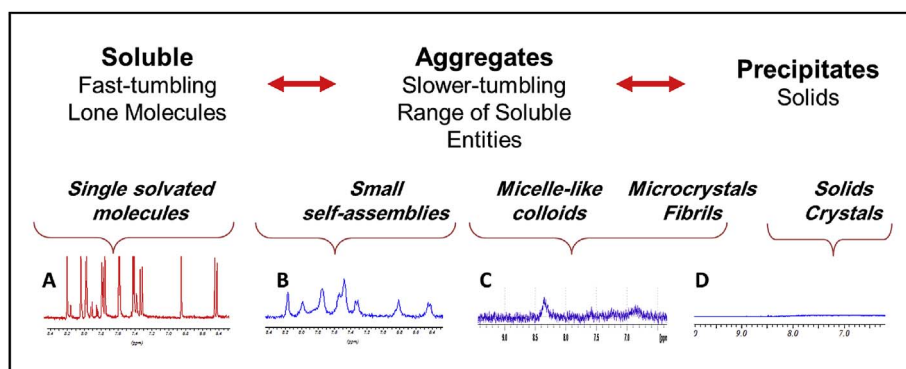


Fig. 1. Compounds can adopt a three-phase equilibrium when placed in aqueous media. On the bottom are ^1H NMR spectra (600 MHz) of various compounds in buffer (50 mM sodium phosphate pH 7.4) at nominal concentrations of 200 μM .

0), Allura Red (25956-17-6), Fast Green (2353-45-9), Erochrome Blue Black B (3564-14-5), Naphthol Yellow (846-70-8), Sudan II (3118-97-6), Acid Violet (1694-09-3), Indigo carmine (860-22-0), Methylene blue (61-73-4), Tartrazine (1934-21-0), Solvent Orange 2 (2646-17-5), 4,4'-(9-Fluorenylidene)dianiline (15499-84-0), 4-((4-hydroxy-1-naphthalenyl)azo)benzenesulfonic acid, monosodium salt (523-44-4), Fast Green (2353-45-9), Quetiapine fumarate (111974-72-2). All were purchased from TCI whereas Acid Green (3087-16-9) and Patent Blue (3536-49-0) were purchased from Chem-Imp.

2.2. NMR sample preparation

Powder dyes were weighed and appropriate amounts placed in Eppendorf tubes followed by the addition of deuterated DMSO to form 20 mM dye stock solutions, as described in Fig. 2. The buffer used for preparing the NMR samples was 50 mM sodium phosphate pH 7.4 in

100% D_2O . When preparing the aggregation buffer, note that a pH of 7.8 corresponds to a pH of 7.4. Tween 80 stock (10% vol/vol in above buffer) was added to samples as defined in the procedure provided below. Further details on how samples were prepared are provided in Fig. 2.

2.3. NMR experiments

The pulse programs for the NMR aggregation assay are the standard one-dimensional ^1H NMR experiments available on all commercial spectrometers. There are several optional parameters that can be modified if desired. Given that the buffer consists of 100% D_2O , one can choose to use a standard ^1H NMR pulse program or one that includes solvent suppression. The latter may be desirable if large H_2O resonance peaks exist due to the hygroscopic property of deuterium oxide. The experiments shown here were run on a 600 MHz Bruker AV III NMR

Sample Preparation for NMR Aggregation Assay

Required Supplies:

- Aggregation Buffer:
 - 50 mM sodium phosphate pH 7.4 in 100% D_2O
- Tween 80 stock (10 % vol/vol in above buffer)
- 7 Eppendorf tubes
- 2 Pipettes: 2 – 20 μL and 200 – 1000 μL

Calculation for 20 mM compound stock solution:

X = exact mass weighed in mg (0.3 – 0.6 mg)

Y = MW of your compound

Z = volume of DMSO- d_6 to be calculated / added

$$\frac{X \text{ mg}}{Y \text{ mg/mmol}} = Z \text{ mL} \quad \left[\begin{array}{l} \text{Volume should work} \\ \text{out to 20 – 200 } \mu\text{L} \end{array} \right]$$

A- Prepare 20 mM compound stock solution (as above)

B- Portion out aggregation buffer

- 1500 μL in tube 5
- 500 μL in each of tubes 1-4

C- Add/mix 15 μL of above 20 mM compound stock to tube 5

- This tube now becomes the 200 μM stock solution
- If precipitate forms, take note and proceed

D- Transfer 500 μL of the 200 μM stock solution to tube 6.

E- Take 500 μL of the 200 μM stock and add to tube 4

- Mix with the 500 μL buffer already present
- This becomes first 2x dilution
- Transfer 500 μL from tube 4 to tube 3. Mix.
- Repeat dilution and mixing for tube 2 then 1.

F- Lightly centrifuge tube 6 (2,000 rpm 10 minutes).

Transfer precipitate-free supernatant to a spare tube

G- Add/Mix 8 μL of Tween 80 stock solution to a spare tube

H- Transfer solution from tubes 1-5 and spare tube to separate NMR tubes

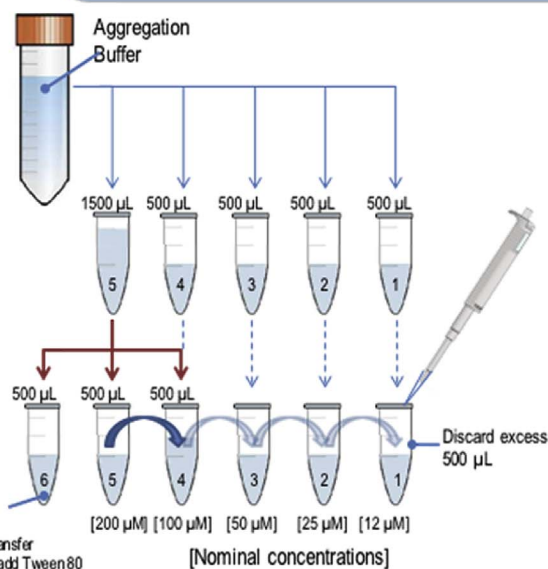


Fig. 2. Detailed procedure for preparing samples for the NMR aggregation assay [1,2].

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