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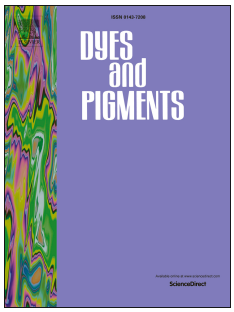

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PII: S0143-7208(17)31728-X

DOI: 10.1016/j.dyepig.2018.02.026

Reference: DYPI 6558

To appear in: *Dyes and Pigments*

Received Date: 11 August 2017

Revised Date: 11 February 2018

Accepted Date: 16 February 2018

Please cite this article as: Murugesan JR, Shahout F, Dlim M, Langella MM, Cuadra-Foy E, Forgione P, LaPlante SR, Revealing dye and dye-drug aggregation into nano-entities using NMR, *Dyes and Pigments* (2018), doi: 10.1016/j.dyepig.2018.02.026.

Revealing Dye and Dye-drug Aggregation Into Nano-entities Using NMR

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Keywords

Dye
Drug
Aggregation
NMR
Nano-entities
Colloids

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 – 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).

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 (Figure 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.

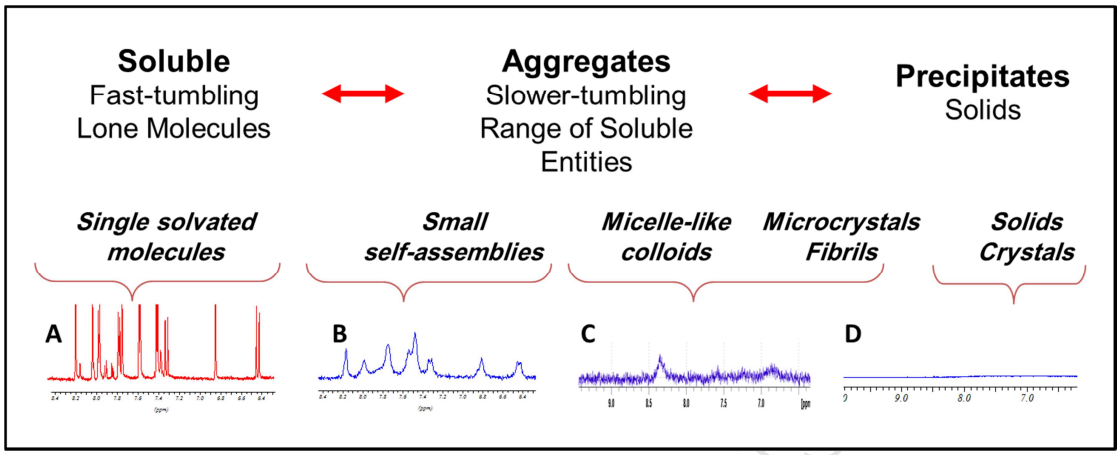

Figure 1. Compounds can adopt a three-phase equilibrium when placed in aqueous media. On the bottom are ^{1}H NMR spectra (600 MHz) of various compounds in buffer (50 mM sodium phosphate pH 7.4) at nominal concentrations of 200 μM.

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 microsope 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. Figure 1 shows how NMR is sensitive to compound tumbling rates and behavior for all three phases. That is, fast tumbling molecules exhibit sharp resonances (Figure 1A), and solid precipitates result in extremely broad and unobservable resonances by solution NMR (Figure 1D). Aggregates give rise to intermediate resonance attributes such as broad resonances and/or unusual features such as those shown in Figure 1B and 1C. 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.

MATERIALS AND METHODS

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 (16423-68-0), Allura Red (25956-17-6), Fast Green (2353-45-9), Erochrome Blue Black B (3564-14-5), Napthol Yellow (846-70-8), Sudan II (3118-97-6), Acid Violet (1694-09-3), Indigo carmine (860-22-0), Solvent Orange 2 (2646-17-5), 4,4-9-Fluorene (15499-84-0 4-((4-hydroxy-1-naphthalenyl)azo)benzenesulfonicacid,monosodiumsalt (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.

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 Figure 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 Figure 2.

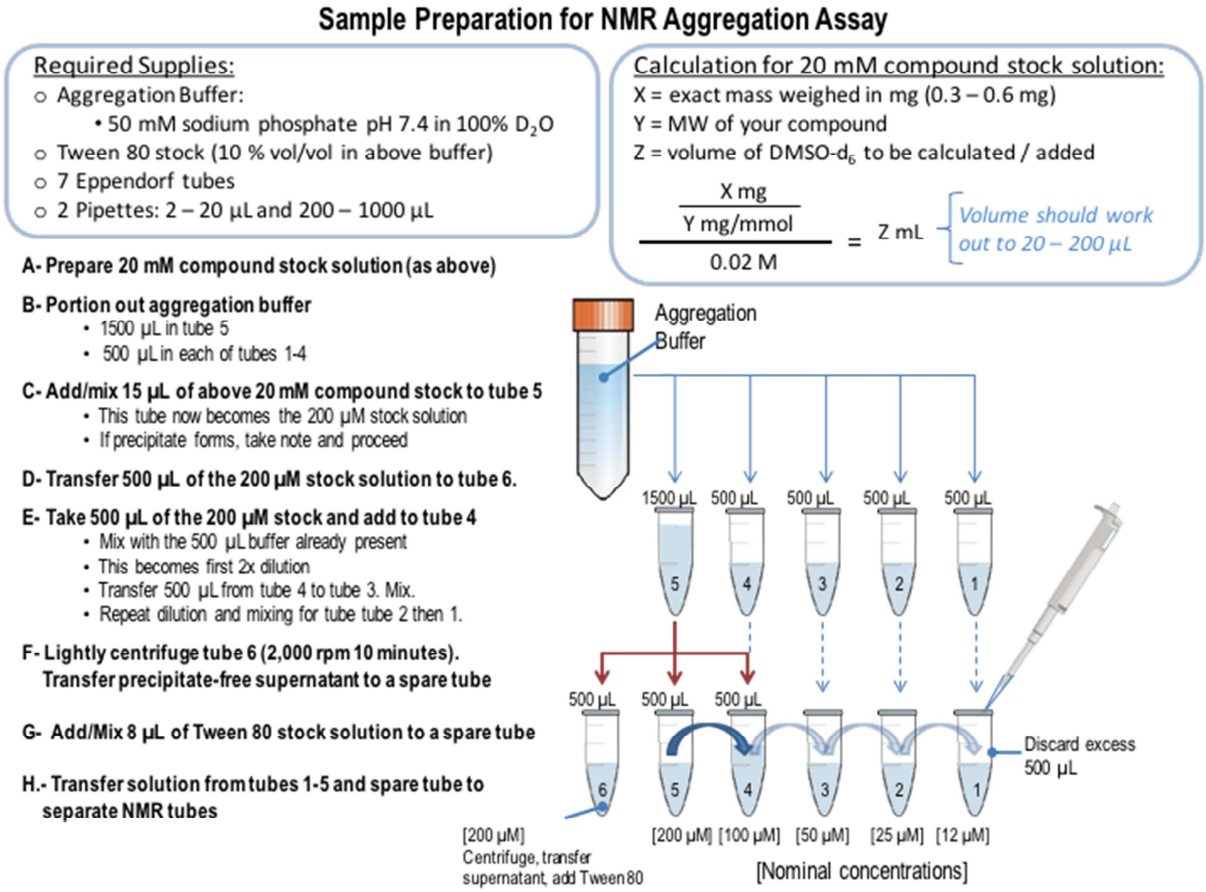

Figure 2. Detailed procedure for preparing samples for the NMR aggregation assay [1-2].

NMR experiments. The pulse programs for the NMR aggregation assay are the standard one-dimensional ^{1}H 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 ^{1}H 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 equipped with a sample changer. The number of scans was typically 256 scans, with a relaxation delay plus acquisition time of 2s, which ensured that all samples for each compound could be, acquired overnight using a sample changer. Data visualization and

interpretation are also simple. For the work described here, Bruker's TOPSPIN software allows for the facile superposition of 1D NMR spectra along with zooming capabilities. Other software from ACD and other vendors also allow for spectral superpositions. Instant JChem for Office Version 18.3.0 was used for chemical structure handling, data analyzing, visualizing and reporting capabilities within the Microsoft Office environment Chemaxon (https://www.chemaxon.com) [4].

Electron Microscopy. Congo Red was diluted in DMSO with 50 mM sodium phosphate pH 7.4 in 100% D_2O, and was prepared at 600 µM. Briefly, 100 µL of the sample was transferred into a 240 µL Airfuge tube. A carbon coated copper grid was inserted into the bottom of Airfuge tube with fine tweezers and centrifuged for 5 min at 20 psi. With tweezers, the carbon grid was gently removed and washed with distilled water for 1 minute. The carbon grid was then negatively stained with 3% of phosphotungstic acid (PTA-3) for 1 min. The grid was then removed and blotted to dry with a bibulous paper and examined with a transmission electron microscope (Hitachi H-7100). The photographs were processed with the digital camera AMT version 600.147.

RESULTS AND DISCUSSION

Detection of aggregation using ^{1}H NMR spectra as a function of dye concentration. The NMR aggregation assay involves the acquisition and monitoring of ^{1}H NMR spectra as a function of compound concentration. For single molecules that do not aggregate, each dye molecule is distal to one another, tumbles freely and is not affected by dilution. Sharp resonances are expected at all concentrations and do not shift left or right. Also, there should be no changes in the number and shape of the resonances (Figure 3). The only changes expected are the

resonance intensities as a function of concentration. For dyes that aggregate the molecules self-associate at higher concentrations, upon dilution they become more distal and tumble more freely. As a result, unusual NMR features and changes are expected due to changes in local environments (Figure 3).

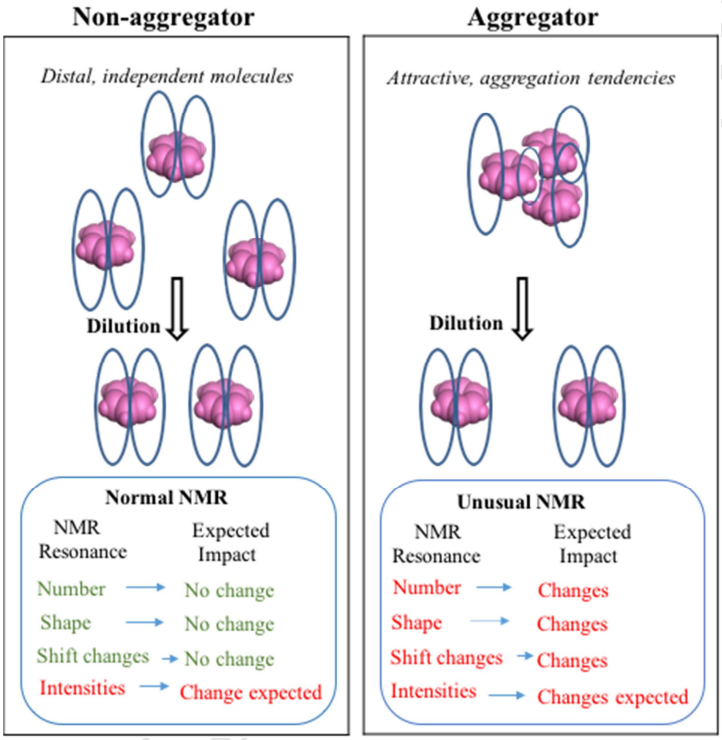

Figure 3. Overview of the expected observations of NMR spectra and resonances upon dilution for dyes that aggregate versus those that do not in solution.

NMR assay on structurally different dyes. Figure 4 displays the ^{1}H NMR spectra as a function of concentration for Tartrazine, Methylene blue and Congo red. An overview of the spectra for Tartrazine suggests that the data is consistent with the predominance of the non-aggregating phase consisting of lone-molecules tumbling rapidly in solution. A single set of resonances exist which are sharp at all concentrations and are well-aligned (no shift left nor right). Furthermore, the addition of Tween 80 had no effect on the resonances intensities, which

is expected in the absence of large, micellular aggregates. Electron microscopy also shows no signs of aggregates (Figure 4).

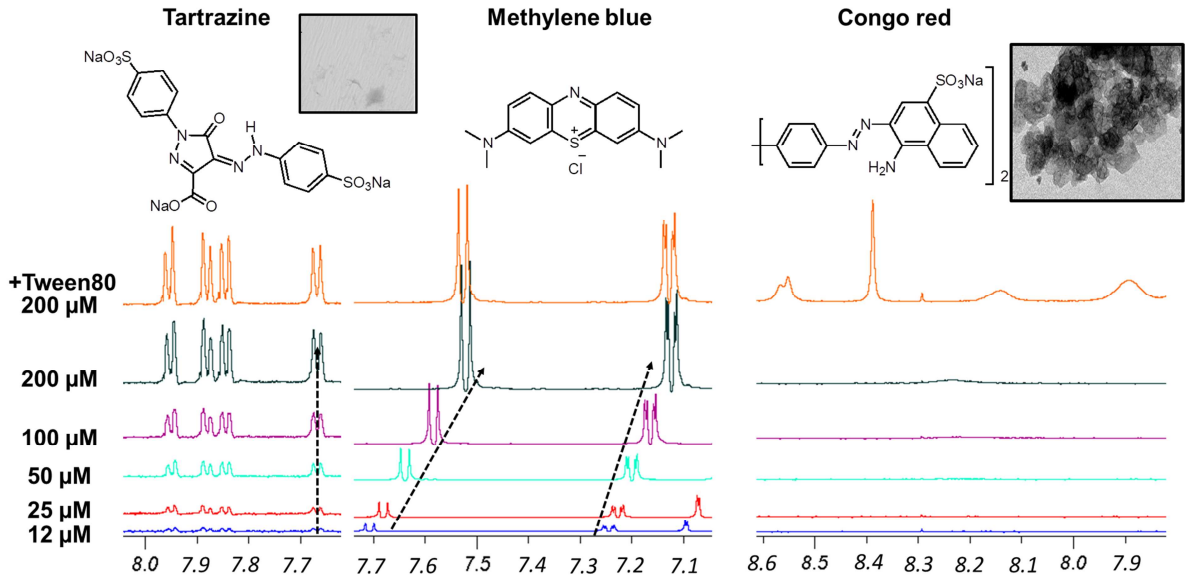

Figure 4. NMR aggergation assay showing ^{1}H NMR spectra of three structurally different dyes obtained by dilution from 200 µM to 12 µM. Data displayed for Tartrazine (left), Methylene blue (middle) and Congo red (right). The insets on the top-right and left are electron micrographs of Congo red and Tartrazine in solution, respectively.

Alternatively, Methylene blue and Congo red exhibit unusual spectral trends that are consistent with the observation of aggregates. Upon increasing the concentration of Methylene blue, the resonances shift significantly indicating changes in local environments upon self-association. These nano-entities are relatively small given the fact that resonances are observed, likely multimeric forms. This is in stark contrast to the observations made for Congo red. No sharp resonances are readily observed at all concentrations, despite exhibiting full solubility (clear solution, precipitate-free). Thus, Congo red self-assembles into very large nano-entities that result in extremely broad, unobservable resonances. A closer look at the 200 µM concentration shows very broad peaks (*vide infra*), which become much sharper but nonetheless

relatively broad upon addition of Tween-80. This latter observation suggests that Tween 80 partially breaks apart the Congo red aggregates into smaller entities that are finally observable as shown in Figure 4. Congo red was also reported [5-7] to form large aggregates and here we show this by scanning electron microscopy (see electron micrograph on the top right of Figure 4).

The scope of the current study was expanded to other classes of dyes, naphthol yellow S hydrate, Erythrosine B and 4, 4'-(9-fluorenylidene) dianiline (Figure 5). Naphthol yellow S (hydrate) exhibited sharp resonances with no shift changes upon increasing the concentration, which is consistent with this dye behaving predominantly as a non-aggregator. For Erythrosine B and 4,4'-(9-fluorenylidene) dianiline, unusual features were observed in ^{1}H spectra of the NMR assay that were consistent with self-assemblies. ^{1}H NMR signals shifted right at higher concentrations of Erythrosine B, and resonances of 4,4'-(9-fluorenylidene)dianiline were only notable upon addition of Tween 80.

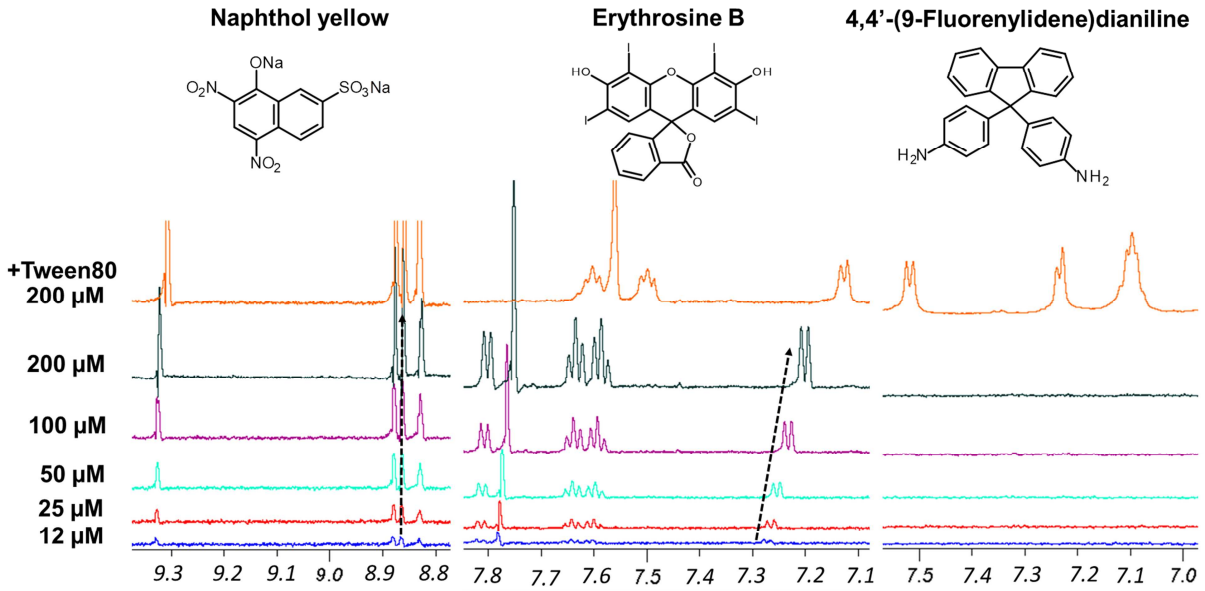

Figure 5. NMR aggergation assay showing ^{1}H NMR spectra of three structurally different dyes obtained by dilution from 200 µM to 12 µM. Data displayed for Naphthol yellow (left), Erythrosine B (middle) and 4,4'-(9-fluorenylidene)dianiline (right).

Other types of dyes were also evaluated (see Supporting Information, Figures S1-S2). For example, Figure S1 shows that Acid blue 9, Evans blue, and Indigo carmine also form distinct types of nano-entities. In the spectra of Acid blue 9, the ^{1}H NMR signals appear as expected for a non-aggregator, except that additional small resonances appear at higher concentrations. More apparent and unusual features are noted in the spectra of Evans blue and Indigo carmine.

NMR assay on structurally similar dyes - Triarymethane class. We next explored the aggregation tendencies of a series of similar compounds. Overall, it was noted that dyes of the same class with subtle structural differences can show completely different aggregation tendencies. This is exemplified in Figure 6 for three triarylmethane dyes (Patent blue, Light green SF yellowish and Acid violet 49). On one hand, the spectra of the Patent blue appears such that no aggregation is observed since no changes in the resonance number, shape and chemical shift occurs at the various concentrations. However, the spectra of Light green SF yellowish and Acid violet 49 display notable unusual trends. The resonances of Light green SF Yellowish exhibit shifts and the presence of two sets of peaks, which is indicative of the presence of more than one type of aggregate species. Broad resonances are observed for Acid Violet 49. These findings are consistent with the literature of cyanine dyes that are reported to adopt J and the H aggregates (bathochromic shift and hypsochromic shift in the absorption spectra, respectively) [8].

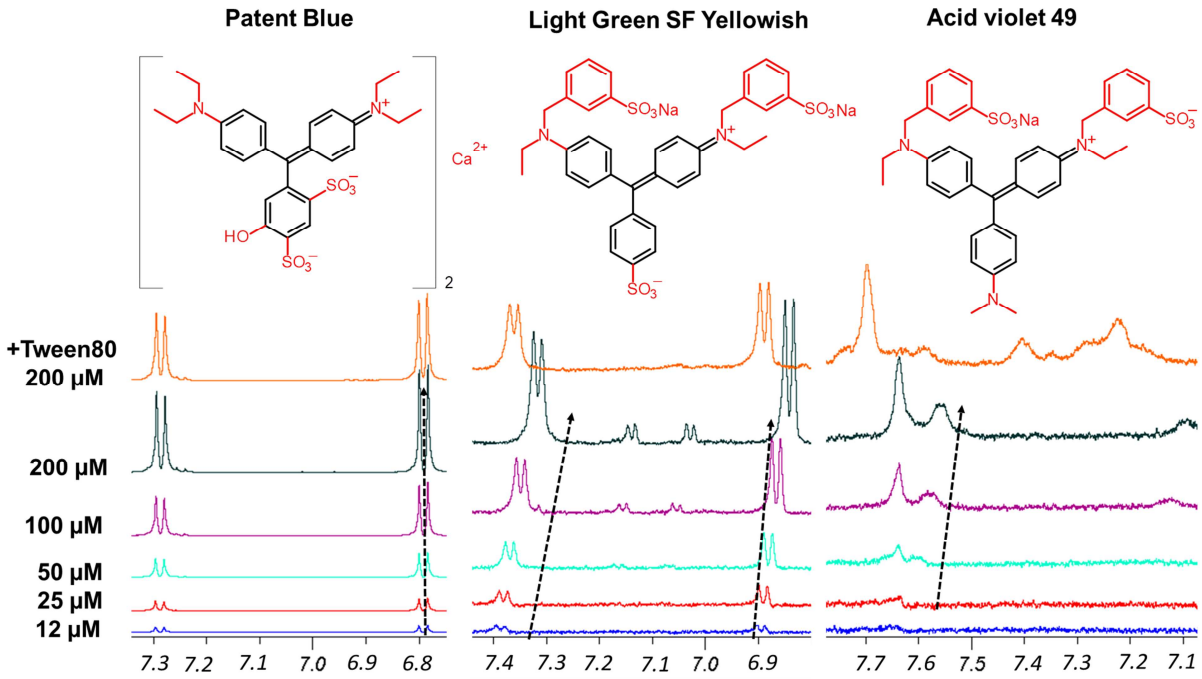

Figure 6. NMR aggergation assay showing ^{1}H NMR spectra of three structurally similar triarylmethane dyes obtained by dilution from 200 µM to 12 µM. Patent blue (left), Light green SF yellowish (middle) and Acid violet 49 (right).

Perhaps the latter two dyes tend to aggregate as a result of π-π stacking interactions of the planar solvophobic aromatic rings, [9] whereas Patent blue has the tetrahedral sulfonate groups in both the *ortho* and *para* positions of a phenyl ring that could prevent such π-π interactions. For Light green SF yellowish and Acid violet 49, the substituents are far away from the aromatic system (*para*) which may minimize steric influences and allow self-assembly.

NMR assay on structurally similar dyes - *Azo dyes with one phenyl and one naphthyl and bis-naphthyl group.* The aggregation tendencies of another series of structurally related dyes was explored to see if the above observations were specific to one series or general. Figure 7

displays the NMR data for three structurally-related azo dyes (Sunset yellow, Allura red and Orange II). In this case, all three compounds have unusual ^{1}H NMR spectral tendencies. The resonances of Sunset yellow are sharp but shift as a function of higher concentration – which is consistent with the existence of small nano-entities and literature reports using other spectroscopic studies [10-12]. For Allura red, the spectra contain both sharp and broad resonances indicating the presence of a mix of multiple aggregate types. On the other hand, the spectra for Orange II are consistent with the presence of very large nano-entities given that resonances only become apparent upon the addition of detergent. Clearly, these results suggest that structure-aggregate relationships exist and that the sulfonate anions play a more complex role beyond the simple expectation of enhancement of solubility [13].

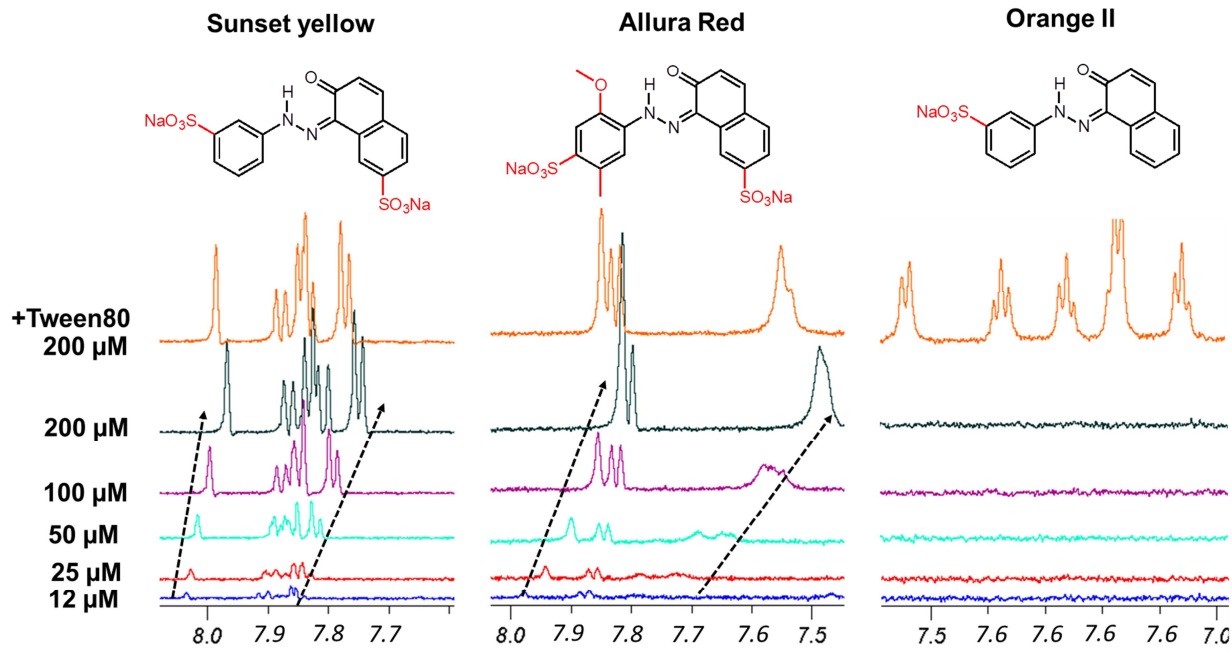

Figure 7. NMR aggergation assay showing ^{1}H NMR spectra of three structurally similar azo dyes obtained by dilution from 200 µM to 12 µM. Sunset yellow (left), Allura red (middle) and Orange II (right).

The structure-aggregation relationships exposed above was then expanded to the structurally similar bis-naphthyl azo dyes that also have a different number of sulfonate anions.

Figure 8 reports the ^{1}H NMR spectra of Acid red 18, Amaranth and Eriochrome blue black B. A single set of resonances of Acid red 18 are observed and sharp throughout the concentration range, although there are some notable resonance shifts at higher concentrations. More dramatic shifts are found for Amaranth that is a regioisomer of Acid red 18. Perhaps the less pronounced aggregate behavior is notable for Acid red 18 due to a suppression of π-π stacking interactions given the proximity of the sulfonate groups to both naphthyl rings, as compared to Amaranth where the sulfonate anions lie at the periphery of the rings. The spectra of Eriochrome blue black B have truly unusual features with many sharp resonances that are inconsistent with a single species.

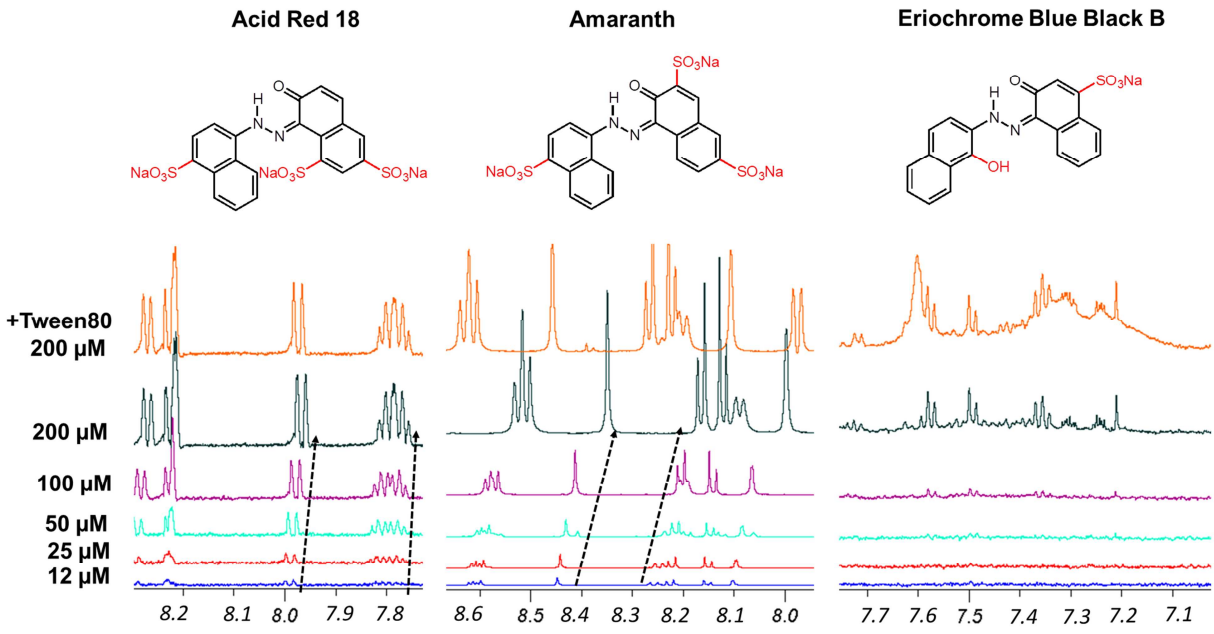

Figure 8. NMR aggergation assay showing ^{1}H NMR spectra of three structurally similar bis-napthyl azo dyes obtained by dilution from 200 µM to 12 µM. Acid red 18 (left), Amaranth (middle), and Eriochrome blue black B (right).

Dye-Drug interactions. Dyes are used in a wide range of applications, including foods, drinks, cosmetics, tooth paste, medications. etc., Thus, a question worth addressing is whether dyes can interact directly with medical drugs that are also highly prescribed in our society. If so,

there may be a potential that dyes can compromise the intended medical use of certain drugs. Unfortunately, there have been few reports on drugs and other chemicals interacting with dyes to produce undesired effects [14], and as a result a detailed molecular view is lacking.

To illustrate how potential interactions can be studied using NMR, Congo red was added to Quetiapine and the NMR spectrum observed. Quetiapine is an antipsychotic drug used for the treatment of schizophrenia and bipolar disorder. The NMR spectrum of Quetiapine alone in Figure 9 shows sharp resonances that are consistent with a non-aggregator behavior in solution. Congo red, which was used in the cellulose industry before it was banned for toxicity reasons, exhibits very broad resonances in Figure 9 that is consistent with a behavior as an aggregator in solution. When mixed together, the broad resonances of Congo red remain whereas the resonances of Quetiapine become significantly broad. Based on this, it is apparent that Quetiapine interacts with Congo red and adopts its slow tumbling behavior as an aggregate. It also shows the potential impact that aggregates can have on other small molecules. Moreover, this provides a new tool for studying drug-dye or drug-drug interactions.

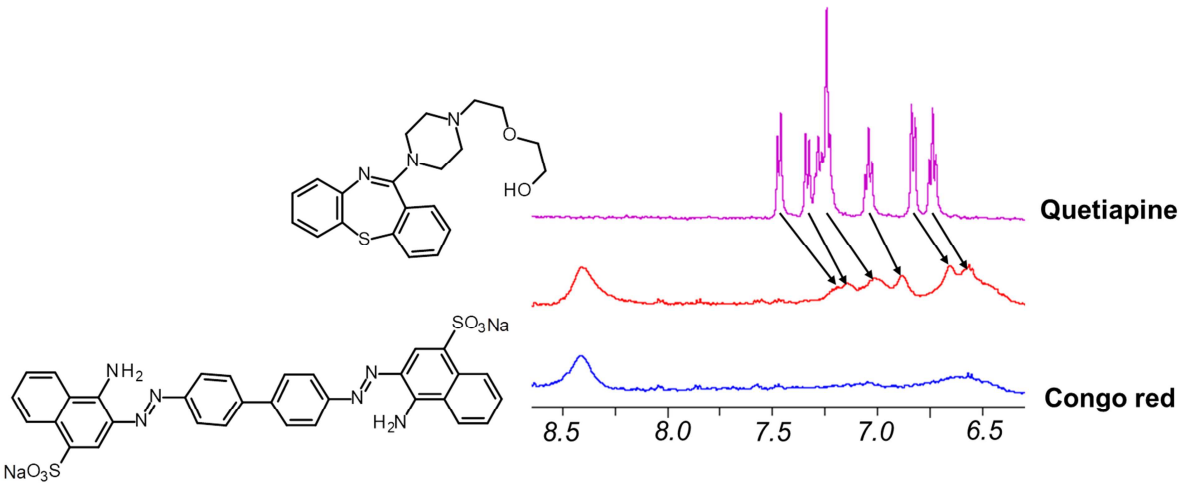

Figure 9. Shown are NMR spectra of the drug Quetiapine and the dye Congo red. ^{1}H NMR spectrum of Quetiapine alone (purple above) at 200 µM. ^{1}H NMR spectrum of 50/50 mixture of Quetiapine and Congo red at 200 µM each (red spectrum, middle) and ^{1}H NMR of Congo red

alone at 200 µM (Blue, bottom spectrum). Note that the broad peaks observed here for free Congo red are less notable in the spectrum of free Congo red in Figure 3 because the vertical scale is much lower. Samples were prepared in 50 mM sodium phosphate buffer pH 7.4 in 100% D_2O solvent.

Discussion on Dyes, Solution Behavior and Properties. Dyes play an important role in our society, and are used in many products. They are produced in extremely large quantities world-wide for applications in various industries. For example, the clothing industry employs dyes to enhance marketability of their products. Also, the food industry employs color to enhance the attractiveness of their products. As a result, consumers are heavily exposed to small-molecule dyes that are worn and ingested, despite the fact that relatively little is known about their *in vivo* behavior, properties and toxicity. The impact on the environment is also significant. Effluents from the dye industry are eventually discharged into the environment as pollutants. Some can be readily degraded, but others, such as azo dyes are persistent as a result of their lipophilic nature. When the latter is degraded, azo dyes are well known to be susceptible to anaerobic reduction, releasing amines and hydrazine that can be carcinogenic [15]. Thus, dyes are highly prevalent in our society and their properties need to be investigated with appropriate tools.

Here, we introduce an NMR aggregation assay and found that some dyes tend to behave as lone single molecules in solution whereas others adopt nano-entity features. It is interesting that we also demonstrated that minor chemical changes can result in major differences in solution behavior. Our previous work, [2] suggested that these solution behaviors can have a serious impact on properties (e.g. toxicity) so it is reasonable that dyes having different behaviors can also have distinct properties. This NMR assay now provides a new tool for monitoring the behavior of dyes in solution, and can begin to explore potential correlations with relevant properties.

Correlating nano-entities to specific dye properties will certainly prove to be difficult or even impossible. However, it is tempting to hypothesize that aggregate-property relationships can exist. For example, the aggregation property of an HIV drug has been directly attributed to its high and favorable oral bioavailability [16]. Also, aggregates have been associated with affecting the efficacy of cancer drugs [17-18]. In a 2007 conference in Southampton UK, the use of six dyes (Tartrazine, Sunset Yellow, Allura Red, Acid Red 18, Azorubine and Quinoline Yellow WS) was questioned because they were suspected of causing food intolerance and exasperating attention deficit hyperactivity disorder (ADHD) in children [19]. Interestingly, this study showed that four of the six dyes exhibit nano-entity properties. For example, Azorubine clearly aggregates in our assay as shown in Figure 10. Other potential properties of dyes are beginning to become evident. For example, some artificial coloring agents appear to aggravate attention deficit hyperactivity disorder (ADHD), and it has been established that erythrosine-based food coloring can cause thyroid tumors in rats. Although little is known about the precise mechanism of toxicity of some dyes, many have been banned (e.g. 42 benzidine and 70 azo dyes) [20].

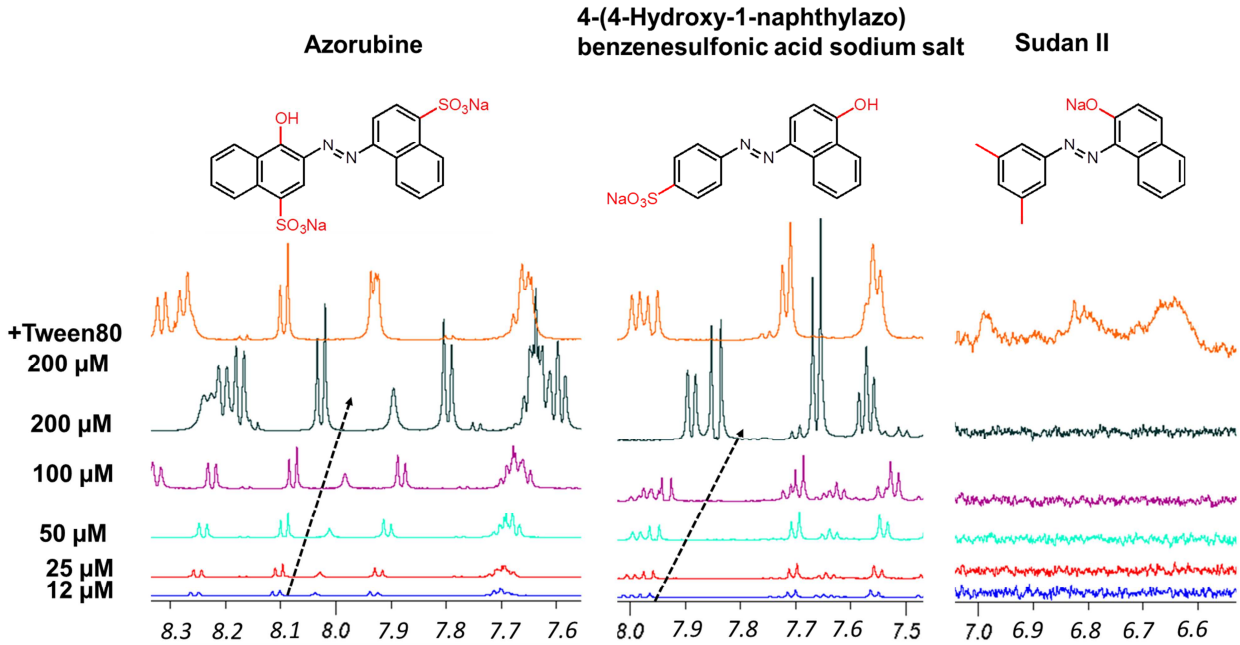

Figure 10. Portions of the superimposed ^{1}H NMR spectra of three structurally similar azo dyes obtained by dilution from 200 µM to 12 µM. Azorubine (left), 4-(4-hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt (middle) and Sudan II (right),1-(2,4-dimethylphenylazo)-2-naphthol. Broken arrows indicate changes in chemical shift (δ ppm) with concentration. NMR samples were prepared in 50 mM sodium phosphate buffer pH 7.4 in 100% D_2O solvent.

CONCLUSION

Here we introduced the NMR aggregation assay as a new tool for monitoring the behavior of dyes in solution. One potential utility of this tool is to explore potential correlations with relevant properties. On one hand, we know that dyes have a variety of properties where some are benign and others are toxic and have been banned from human consumption. On the other hand, we also now know that some dyes can have a variety of aggregate behaviors, and aggregates have been shown to exhibit promiscuous properties and even toxicity. Although focused studies will be needed to properly establish behavior-property correlations, the NMR methods shown here may be a good method to provide new insights.

NOTE

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors wish to acknowledge Anne-Laure Larroque and Sanjoy Kumar-Das of the McGill University Health Centre (MUHC) for kindly providing 600 MHz NMR time. They also thank Norman Aubry and Rebekah Carson for helpful discussions and insight. Technical support was provided by Sagar Saran and Paul Oguadinma.

SUPPORTING INFORMATION

Supplementary data related to this article can be found at http:// dx.doi.org/XXXX/j.dyepig.2017.GG.CC

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SUPPORTING INFORMATION

The NMR aggregation was employed to other dyes and the displays of the NMR data are shown below as Figures S1 and S2.

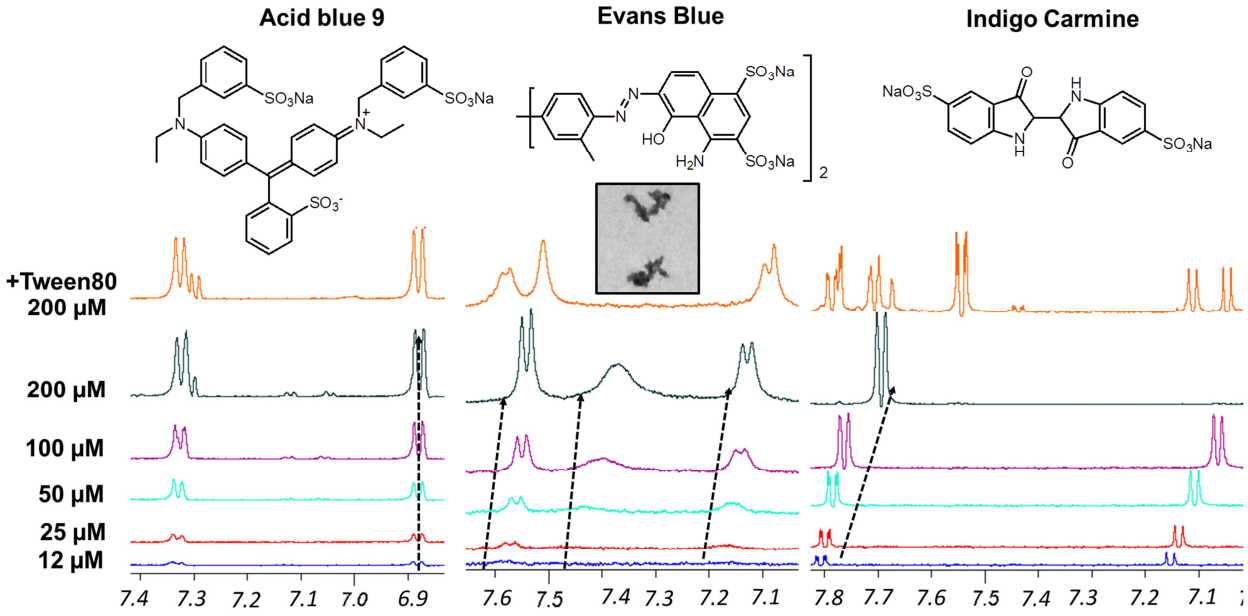

Figure S1. Portions of the superimposed ^{1}H NMR spectra of three structurally different dyes obtained by dilution from 200 µM to 12 µM. Acid blue 9 (left), Evans blue (middle), both medium-sized and Indigo carmine (right). Broken arrows indicate changes in chemical shift (δ ppm) with concentration. NMR samples were prepared in 50 mM sodium phosphate buffer pH 7.4 in 100% D_2O solvent.

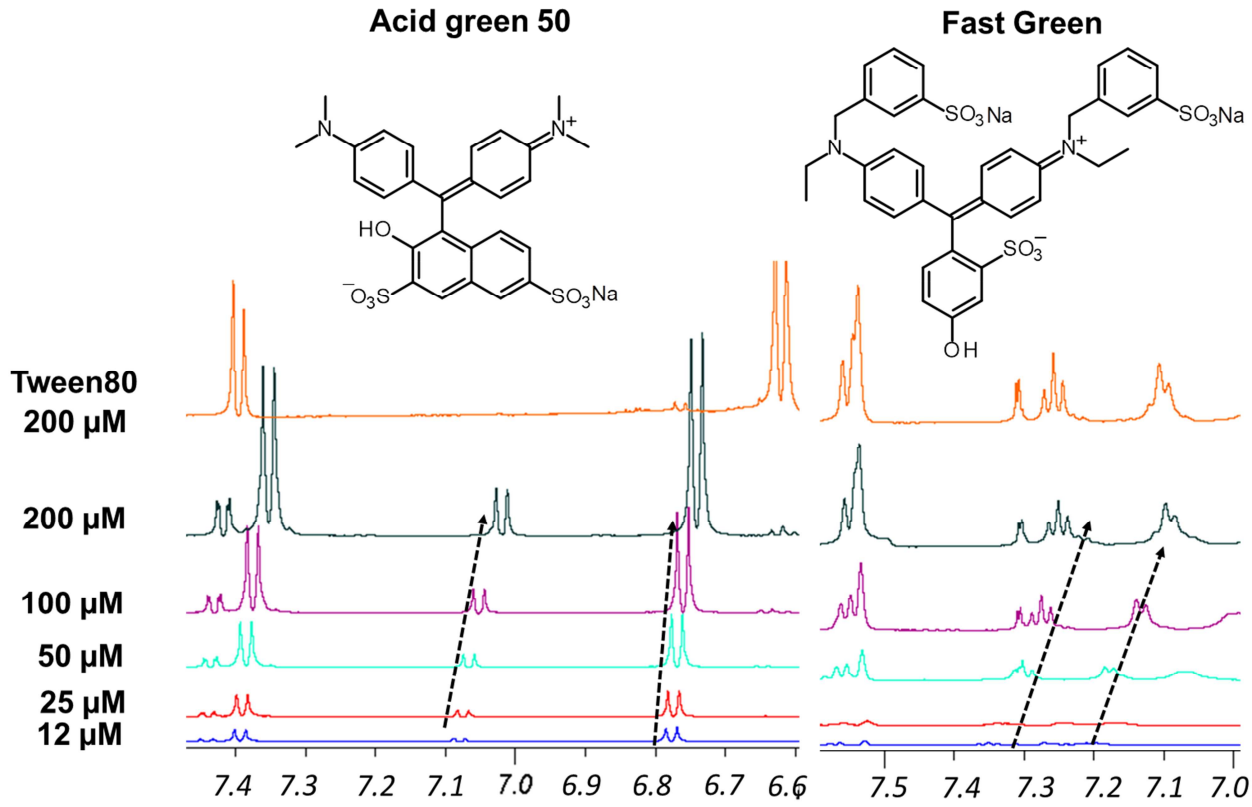

Figure S2. Portions of the superimposed ^{1}H NMR spectra of Acid Green 50 (left) and Fast Green (right) dyes obtained by dilution from 200 µM to 12 µM. Broken arrows indicate changes in chemical shift (δ ppm) with concentration. NMR samples were prepared in 50 mM sodium phosphate buffer pH 7.4 in 100% D_2O solvent.