A rapid colorimetric method to visualise protein interactions

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Abstract: As key molecules in most biological pathways, proteins physically contact one or more biomolecules in a highly specific manner. Several driving forces (i.e. electrostatic and hydrophobic) facilitate such interactions and variety of methods have been developed to monitor these processes both in vivo and in vitro. Here, we propose a new method for the detection of protein interactions by visualizing a colour change of a cyanine compound, a supramolecule complex of 3, 3' di (3-sulfopropyl) - 4, 5, 4', 5'-dibenzo- 9-methyl - thiacarbocyanine triethylam - monium salt (MTC). Nuclear Magnetic Resonances (NMR) studies suggest that the hydrophobic nature of the protein surfaces drives MTC into different types of aggregates with distinct colors. When proteins interact with other biomolecules, the hydrophobic surface of the complex differs, resulting in a shift in the form MTC aggregation which results in a color change. As a result, this in vitro method has the potential to become a rapid tool for the confirmation of protein–biomolecule interactions, without the requirements for sophisticated instrumentation or approaches.

Proteins, the final products of the genetic code, execute most biological tasks inside the cell. They can be structural components supporting the architecture of the cell, enzymes that catalyze chemical reactions, messengers that transmit signals to coordinate biological processes, antibodies that provide defense for the body, and many other roles. Proteins rarely work alone and they need to physically interact with various molecules to conduct their molecular activities. A variety of methods have been developed to detect protein interactions, for example in vivo two hybrid[1] assays and in vitro isothermal titration calorimetry[2]. Currently, most common laboratory practices for the identification of protein interactions are chromatography or electrophoresis based, e.g. pull downs/SDS PAGE for protein-protein interaction and mobility shift assay for protein-nucleic acid interaction. The success of these methods is dependent on interactional affinity and require specific experimental setups. A simpler, quicker and universal approach would not only benefit biological research, but may also accelerate large-scale ligand screening.

Herein, we sought to develop a rapid determination method based on the supramolecular assembly of certain cyanine dyes. Although branded as one of the oldest classes of synthetic compounds, cyanine dyes are still finding new applications. One of the most intriguing findings is that it can bind non-covalently to helical structures of oligonucleotides and exhibit a significant fluorescence enhancement[3]. The applications of these dyes have been exploited in staining DNA[4] and RNA G-quadruplexes (G4s) structures inside cells[5, 6]. Another interesting feature of these dyes is a responsive colorimetric indicator of its supramolecular assembly. Under favourable conditions the supramolecule switches form, between J- and H-aggregate, which is accompanied by observable changes in absorbance spectra and colour[7]. Short-range, non-covalent forces, including van der Waals and π-π stacking interactions, contribute to the aggregate formation[8]. Many detection methods have been developed based on the colorimetric properties[9-11]. More recently, one of the cyanine derivatives, MTC, extended its biochemical application to protein folding, by working as conformational indicator of human transferrin[12]. However, these studies were limited to the status of a single molecule rather than oligomeric protein complexes.

Inspired by these studies, we investigated the mechanism behind the protein-dye interaction using solution NMR[13, 14]. We chose proteins samples readily available from our laboratory and others deposited in the Protein Data Bank (PDB). Among those candidates, a bacteriophage protein p7 of Xanthomonas Oryzae for which the NMR spectrum is fully assigned and its solution NMR structure is available[13, 14]. Its ability to form a complex with a small peptide from its binding partner RNA polymerase gave us the opportunity to explore the behavior of MTC in a two-partner system. To provide further insight, we applied MTC to two more systems, one involving a protein-DNA interaction and another involving an interaction between two globular proteins. Our experimental data suggests that the MTC-target protein interaction is driven by hydrophobic forces and the geometry of protein interactional surface provide the basis for specific MTC assembly. Here, we propose a new qualitative method capable of detecting the interactions between protein and multiple types of ligands using a visible colorimetric indication.

Mechanism behind MTC-protein interaction

Due to the extended planar π-electron-conjugated system, MTC is capable of assembling into distinct supramolecular aggregates, either spontaneously or when induced by other molecules. While these aggregates are stabilized by Van der Waals forces, precise how these initiate supramolecular complex formation remains unclear. To understand this further, we used NMR to map the interaction surface on the protein during the process of supramolecular complex formation of cyanine dye (Figure 1). The

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Xanthonomas oryzae protein P7 was chosen to be the target due to its stability in a wide range of buffers and the availability of full NMR assignments[13, 14]. Importantly, P7 is able to tolerate the organic compound MTC at molar concentrations without aggregation or losing structural integrity.

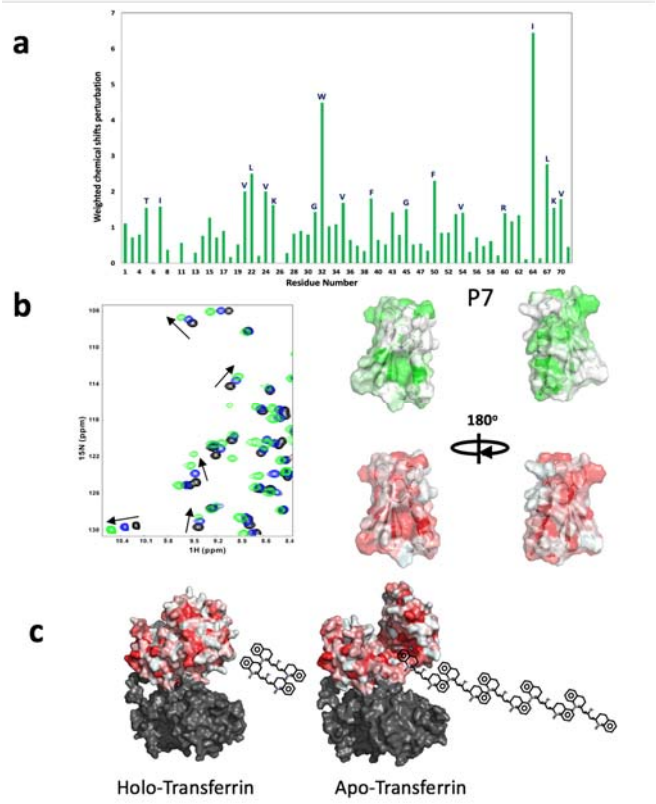

Figure 1. (**a**) The weighted chemical shift perturbation plotted against residue number. Residues with values larger than 1.4 are labelled.B (**b**) Left: Overlay of 2D $^{1}H–^{15}N$ HSQC spectra of the P7 (black). P7 with 5 equivalents of MTC (blue) and P7 with 10 equivalents of MTC (green) and the arrow shows the direction of titration. Top right: chemical shift perturbation mapping of P7 based on NMR titration. Residues affected the most upon adding MTC are colored green while the least/not affected is colored white. Residues with $\Delta\delta > 1.4$ are colored in green, residues with $0.3 < \Delta\delta < 1.4$ are in light green and residues with $\Delta\delta < 0.3$ are in white. Bottom right: the surface of P7 is colored according to the hydrophobicity of individual residues, i.e. least hydrophobic residues are colored white while the most hydrophobic residues are colored red. (**c**) Proposed model for supramolecular assembly in Cyanine -Transferrin interaction: Left, MTC forms dimer with/without presence of holo-transferrin. Right, MTC forms J-aggregates whose nucleation starts from the opening jaw of apo-transferrin. The surface of N terminal of transferrin which is identical in both apo- and holo- is coloured grey and the C terminal is colored according to the hydrophobicity of individual residue. PDB ID for P7: 2MC6, apo-transferrin: 2HAU and holo-transferrin: 3QYT.

NMR titration clearly shows that some residues are affected more significantly than others when MTC was added to P7 (Figure 1a). To further illustrate this difference, we normalized the chemical shift perturbation and mapped it onto the P7 structure (Figure 1b). As NMR spectral changes fit well with the distribution of surface hydrophobicity, it suggests that exposed hydrophobic residues are responsible for MTC interaction (Figure 1b). Specifically, it includes surface exposed residues with aromatic side chains like Phe and Trp, as well as hydrophobic aliphatic side chains like Ile, Leu and Val. A small number of hydrophilic or neutral amino acids affected in the presence of MTC but this is likely due to close proximity to hydrophobic residues. For example, in a major hydrophobic pocket of P7, which is the binding site for its ligand, display significant chemical shift changes during the MTC titration, several hydrophilic amino acids in this region also experience changes. Although there is no report specifically on the effect of hydrophobicity on cyanine aggregation, its effect on other nanoparticles and their assemblies has been intensively studied. For example, chiral nanorod assemblies which formed by intermolecular hydrophobic force, is used in detecting attomolar DNA[15]. Similarly, hydrophobicity is the predominant force in varies nanodiscs in many exciting structural biology and nano-biotechnology applications[16-18].

The fact that many hydrophobic aromatic residues are among the highest affected, suggests that π-π stacking play an important role alongside hydrophobicity in this interaction. Interestingly, π stacking also plays important roles in self-assembly of amyloid fibrils, founded in amyloid-associated disorders, such as Alzheimer's disease[19, 20]. Recent study using Proton magic-angle-spinning NMR technique also suggest π-π interaction is predominant force in amyloid aggregation[21]. It is likely that these aromatic residues represent major seeding elements for the MTC assemblies. The surface geometry around these regions may also contribute to defining the type of aggregate formed. It is particularly notable in the case of the reported transferrin-MTC interaction[12], that the difference between apo- and holo-transferrin is a large hydrophobic patch exposed on a semi-opened jaw of the apo state, while the iron-bound holo form is closed. While holo-transferrin induces the dimer of MTC, while the apo form prefers stair-like J-aggregate (Figure 1c). It is conceivable that the open jaw of the apo-transferrin would allow one of the two wings of MTC to bury inside the hydrophobic pocket and the other wing protrude and provide a platform for MTC assembly. Following this assumption, one would suggest that the surface geometry of the jaw region of apo-transferrin would induce the formation of staggered J-aggregates.

MTC as an indicator of protein-DNA interaction

We next explored the application of the MTC dye to detecting a two-molecule system, such as a protein-DNA interaction. A recently reported protein Gp5.7 from the T7 bacteriophage[22]. Gp5.7 interacts with all types of DNA due to a largely positively charged surface, however it binds specifically to particular promotor regions (Figure 2a). These properties of Gp5.7 allows us examine the two different DNA binding properties in the presence of MTC.

When MTC was added to dsDNA fragments of different sizes, the indicative blue color suggests that MTC forms J-aggregates in all cases. Although both MTC-Gp5.7 and MTC-dsDNA also form J-aggregates (Figure 2), the later has a lower absorbance at 650 nm. MTC-Gp5.7/dsDNA(random) shows a similar spectrum, but displays a decrease in this region, which is in line with Gp5.7 interacting with random dsDNA weakly and likely via a nonspecific

electrostatic interaction (Figure 2a). The spectrum of MTC-Gp5.7/dsDNA(T7 A1), in which the complex is specific (Figure 2b), shows a dramatic absorbance shift from 650 nm region to 520 nm. By comparing the hydrophobicity of Gp5.7 and the chemical shift mapping on Gp5.7-DNA titration, it is clear that the DNA binding surface is confined to a large surface hydrophobic patch. Upon binding to specific DNA, the hydrophobicity profile of the complex surface would be altered leading to different nucleation initiation of MTC assembly. While the color difference between MTC-Gp5.7/dsDNA(random) and MTC-Gp5.7 is rather subtle, which may be indication of weak interaction, the MTC-Gp5.7/dsDNA (T7 A1) shows clear spectra shift and a very clear visible color change. These data show that MTC is capable of detecting specific Gp5.7-dsDNA interaction, and it may therefore have the potential of distinguishing different interaction modes in a more optimized experimental setup.

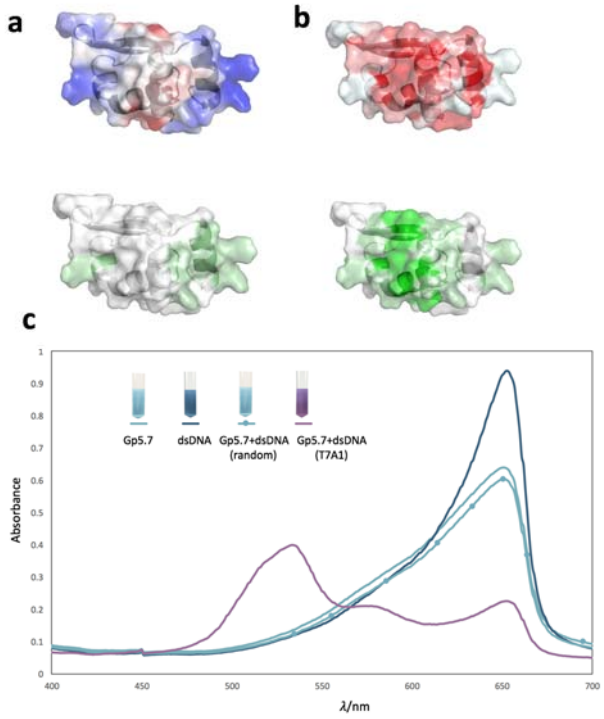

Figure 2. (**a**) Top: electrostatic surface of Gp5.7 (blue: positive charge, red: negative charge). Bottom: Right: DNA binding model 1: random DNA binding due to electrostatic interaction based on NMR chemical shift mapping. (**b**) Top: hydrophobicity of Gp5.7 - least hydrophobic residues are colored white while the most hydrophobic residues are colored red. Bottom: DNA binding model 2: Specific DNA binding in wHTH based on NMR chemical shift mapping. (**c**) Absorption spectra of MTC in different reaction mixtures. The color of the reaction mixture is indicated by the color of the curves and also represented by the test tubes in the legend. Gp5.7/dsDNA ratio is 1:2 as published.

MTC as an indicator in protein-peptide interaction

Here, we add a short target peptide to the previous described protein P7 to probe the assembly behavior of MTC in a protein-peptide scenario. P7 binds to a 10 amino acids long peptide of the RNA polymerase, primarily via a hydrophobic pocket on P7 (Figure 3a and c)[13, 14, 23].

MTC-P7 and MTC-peptide both have absorbance at 650 nm region while the latter also has an additional peak on 470 nm region (Figure 3b). The color of MTC-P7/peptide shows a distinguishable purple color, which is different from that of either MTC-P7 or MTC-peptide. Comparing with monomeric P7 or peptide, the surface hydrophobicity of P7/peptide complex is different as the buried interfaces on both proteins are the major hydrophobic regions (Figure 3c). This example provides further evidence, that the modification of MTC-complex surface hydrophobicity profile affects the type of aggregates that the dye could form.

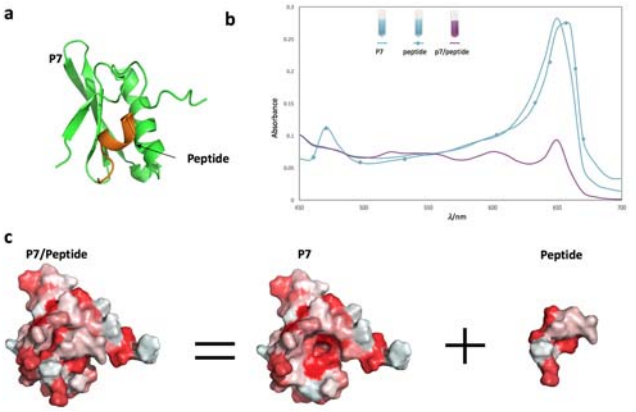

Figure 3. (**a**) Cartoon representation of P7/Peptide complex: P7 green and Peptide Orange. (**b**) Absorption spectra of P7/Peptide, P7 and peptide in presence of MTC, respectively. The color of the reaction mixture is indicated by the color of the curves and also represented by the test tubes in the legend (**c**) Surface hydrophobicity of P7/peptide complex and its components. From left to right: P7/Peptide complex, P7 and peptide. Least hydrophobic residues are colored white while the most hydrophobic residues are colored red.

Protein-protein interaction

To provide more evidence that MTC could be a viable reagent for protein-protein interaction, we chose a well-characterized system from the literature, the Rad18-UBZ/ubiquitin complex (PDB ID: 2MRE), for further investigation[24]. Human Rad18 contains a type 4 UBZ domain and binds ubiquitin with micromolar affinity (Figure 4a).

The spectrum of MTC-Ub suggests that a different type of aggregate is present, while MTC-Rad18 shows mostly J-aggregates. The absorbance of MTC-Ub/Rad18 resembles the spectrum of MTC-Ub with a significant decrease in 650 nm region. Ubiquitin has a very characteristic hydrophobic strip on the side of the protein that partially occupied by Rad18 in the complex (Figure 4c) and augmented by a Rad18 hydrophobic patch. Even though the difference does not seem as dramatic as previous examples, the color changes induced upon binding a clearly identified, suggest that MTC could be used as a general indicator of protein-protein interactions.

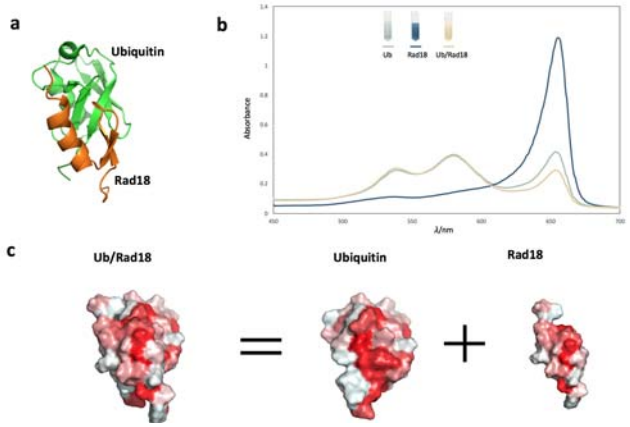

Figure 4. (**a**) Cartoon representation Ub/Rad18 complex: ubiquitin green and Rad18 orange. (**b**) Absorption spectra of Ub/Rad18, ubiquitin and Rad18 in presence of MTC, respectively. The color of the reaction mixture is indicated by the color of the curves and also represented by the test tubes in the legend (**c**) Surface hydrophobicity of Ub/Rad18 complex and its components. From left to right: Ub/Rad18 complex, ubiquitin and Rad18. Least hydrophobic residues are colored white while the most hydrophobic residues are colored red.

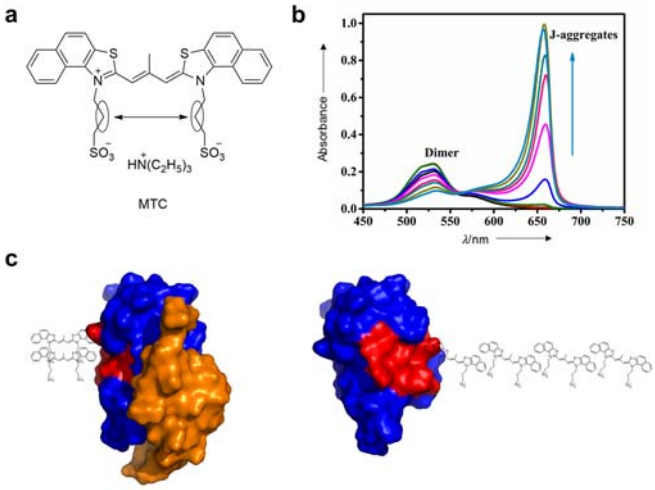

Figure 5. (**a**) Molecular formula of MTC. Arrow indicates the methyl groups that replaced in cETC and PTC. (**b**) Colorimetric difference between different forms of MTC induced by KCl. (**c**) Proposed mechanism of aggregate formation in two partner system. Blue is the candidate protein, red is the major hydrophobic patch on the surface and orange is the ligand (biomolecule). MTC forms J-aggregates with candidate protein by binding to its major hydrophobic patch (upper) and forms dimer with complex on by binding to the major hydrophobic patch on the protein-ligand complex (lower).

Besides MTC, we also tested two other cyanine derivatives, ETC [3, 3' di (3-sulfopropyl) - 4, 5, 4', 5'-dibenzo- 9- ethyl - thiacarbocyanine triethylam - monium salt] and PTC [3, 3' di (3-sulfopropyl) - 4, 5, 4', 5'-dibenzo- 9- phenyl -thiacarbocyanine triethylam - monium salt], in the same set for experiments. While the naphthothiazole ring group remains the same, ETC and PTC has ethyl and phenyl groups in place of the methyl group of MTC, respectively (Figure 5a). Unlike MTC, neither ETC nor PTC was sensitive to the systems tested here as they displayed almost identical spectra for all samples. While the spectrum and color of MTC is affected by ionic strength (Figure 5b), the spectral changes are more complicated when protein surfaces induce the nucleation of aggregation[10]. This Color (spectra)-Aggregate-Surface Hydrophobicity (CASH) relationship allows us to use color to profile protein surfaces. When a protein binds to ligand, the surface hydrophobicity profile will change, at least minimally, and this alteration provides a new nucleation pattern for MTC aggregates.

In summary, we have localized a MTC-P7 interaction to the surface exposed hydrophobic residues using NMR. We propose that the distribution surface hydrophobicity and its geometry are the determining factors for the nucleation of MTC supramolecular assembly (Figure 5c) and this property can be exploited in a simple protein interaction detection method. Furthermore, a more quantitative approach can be taken by measuring OD 650 nm or scanning 400 nm to 700 nm range to obtain the complete profile. It should also be possible to extend this application to other biomolecules, such as carbohydrates and lipids. Such application would make the method universally for all types of ligand with a simple experimental setup. Panacea for all biomolecular interaction problems does not exist and our method may be insensitive in interactions in which hydrophobic profiles remains relatively undisturbed. However, many interactions, especially the ones involve hydrophobic molecules like lipid, would benefit from such simple experiment setup.

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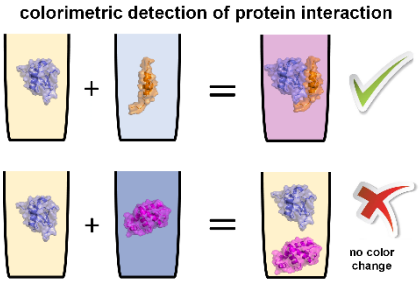

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