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Recent Developments in the Use of Differential Scanning Fluorometry in Protein and Small Molecule Discovery and Characterization

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Abstract

Introduction—Despite tremendous advances in the application of biophysical methods in drug discovery, the preponderance of instruments and techniques still require sophisticated analyses by dedicated personnel and/or large amounts of frequently hard-to-produce proteins. A technique which carries the promise of simplicity and relatively low protein consumption is the differential scanning fluorometry (DSF), wherein protein denaturation is monitored, through the use of environmentally sensitive fluorescent dye, in a temperature-ramp regime by observing the gradual exposure to the solvent of otherwise buried hydrophobic faces of protein domains.

Areas covered—This review describes recent developments in the field, with a special emphasis on advances published during the 2010–2013 period.

Expert Opinion—There has been a significant diversification of DSF applications beyond initial small molecule discovery into areas such as protein therapeutic development, formulation studies, and various mechanistic investigations, serving as a further indication of the broad penetration of the technique. In the small molecule arena, DSF has expanded towards sophisticated co-dependency MOA tests, demonstrating the wealth of information which the technique can provide. Importantly, the first public deposition of a large screening dataset may enable the use of thermal stabilization data in refining *in silico* models for small molecule binding.

Keywords

Differential scanning fluorometry; fluorescence thermal shift; thermal stabilization; chemical chaperones; pharmacological chaperones; inhibitor mechanism of action

1. Introduction

Differential scanning fluorometry (DSF) is a convenient method to evaluate the thermal stability of proteins under a range of conditions, including through the binding of small molecule ligands. Examples of application include determination the melting temperature (T_m) of a purified protein to control the reproducibility of the production process (batch to batch), as a survey method to determine the impact of mutations on protein folding, as a screening tool for best storage conditions, and as a medium- to high-throughput platform to discover small molecule stabilizers of protein targets for drug discovery. The use of DSF in

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mechanism of action (MOA) studies has been accelerating but has received generally less attention. Multiple reviews have been published on the topic of DSF in recent years [1–6]; the present overview will focus on major developments in the technique setup and types of problems it has addressed during the past 3 years.

2. Experimental setup and data interpretation

With respect to physical set up of the DSF experiment, the technique could not be simpler: the protein of interest is mixed with detection dye and candidate stabilizing agents, the sample is heated up in a controlled manner, and fluorescence signal is collected as a function of temperature; no special protein labeling, chip or device preparation, or other sample treatment steps are necessary. The most commonly used dye nowadays is SYPRO Orange whose fluorescence (excitation at ~470 nm/ emission at ~570 nm) is quenched in an aqueous environment. As the temperature rises, the protein undergoes thermal unfolding and exposes its hydrophobic core regions. SYPRO Orange in turn binds to these newly-exposed hydrophobic regions and becomes unquenched. From the resulting fluorescence-versus-temperature plots, the midpoint of the protein unfolding transition is defined as the T_m (melting temperature). Detailed descriptions of the DSF protocol can be found in dedicated publications by the Structural Genomics Consortium [7,8], as well as in multiple studies where the technique is used. Typically, the T_m value can be derived by fitting the data to the Boltzmann equation using publically available analytical tool [7]; more recently, instrument vendors have been providing T_m determination as a standard part of their software. It is important to note that automated T_m determination will only produce reliable values if the thermal transition is uncomplicated, that is, devoid of secondary transitions, noise, or bumps. Because in a typical large-scale DSF experiment one cannot know beforehand whether all melting profiles will be of the simple monophasic kind, the DSF data analysis is always accompanied by some degree of manual examination of the raw thermal melting profiles.

Most of the typical experimental conditions for DSF have already been optimized and are being applied in narrow ranges; these include dye dilution ratio and the temperature ramp rate. Recently, Senisterra *et al.* measured T_m values for nine different proteins under five heating rate conditions (1, 2, 4, 6, and 8 °C/min) and observed that the T_m values generally increased at the faster heating rates. Furthermore, the authors screened the nine proteins against one or two of their known ligands at various concentrations: in the majority of cases, no significant changes in the increases of T_m as a function of heating rate were observed [4].

2.1 Evolution of instruments, thermal regimes, and reporter dyes

DSF was initially introduced, and continues to be used by some, as the Thermofluor method, along with a proprietary instrument [9,10]. During the past decade, a wide range of real-time thermocyclers, as well as plate readers with temperature control, have been utilized to run DSF experiments, including the Stratagene M3005P [11,12], Opticon2 (MJ Research, now Biorad [13] [14]), iCycler/iQ by BioRad (an early example of use by Lo *et al.* [15]), Rotorgene from Corbet Research [16], LightCycler 486 by Roche [17], a range of Applied Biosystems real-time PCR platforms, including the 7900 HT, 7500 Fast and StepOnePlus, and the recently launched ViiA 7 (http://www.invitrogen.com/site/us/en/home/Products-and-

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Services/Applications/PCR/real-time-pcr/real-time-pcr-applications/real-time-pcr-protein-analysis/protein-thermal-shift.html?icid=fr-proteinmelt), and the FluoDia T70 temperature-controlled plate reader by PTI (http://www.pti-nj.com/PlateReader/PlateReader-FluoDia.html). The main variations of the DSF technique are summarized within Table 1.

The environment-sensitive dye initially used in Thermofluor ([10], also see examples of recent use by Clemente et al [9]) was anilinonaphthalenesulfonic acid (ANS), but now SYPRO Orange has become the dominant detection dye of choice; of note, limited studies have shown uniform stabilization reporting by several dyes when tested in parallel [18]. New dyes have also been introduced recently, for example the ProteoStat Protein aggregation assay (Enzo Biochem, extensively benchmarked using native and aggregated IgG) is being used for monitoring peptide and protein aggregation in solution and to optimize buffers and excipients for protein formulation, with claimed sensitivity in the sub-micromolar range and ability to detect as little as 1–5% protein aggregate in a concentrated protein solution (http://www.enzolifesciences.com/browse/bioprocess/proteostat-range-overview/). To monitor protein stability in formulations containing surfactants, a molecular rotor type of fluorescent dye [19] has been validated (presented in detail in 3.1). Notably, vendors such as Life Technologies have launched kits for protein thermal shift assays, in combination with their own line of real-time thermocycler instruments and analysis software; however, it is not clear whether the dyes incorporated in these kits are same as or different from SYPRO Orange.

The majority of investigations using DSF apply the technique as described above, by heating up the sample from room temperature to approximately 95 °C and recording fluorescence as a function of temperature. Variations of this basic protocol do exist, however (Table 1): a study by Senisterra several years ago [20] investigated an isothermal approach to screen for binders of citrate synthase by monitoring time course of fluorescence evolution in the presence of library compounds after bringing the temperature of the samples to approximately 4 °C below the T_m of the protein; good assay stability and applicability to HTS of large libraries was demonstrated.

In yet another variation of DSF, the detection of protein denaturation was changed from direct dye binding to an assay designed to reveal the progressive exposure of hexahistidine tails of the protein molecules (typically incorporated in recombinant proteins for the purpose of affinity purification) upon unfolding: the ATLAS technique (Any Target Ligand Affinity Screen [21,22]) used time-resolved fluorescence resonance energy transfer (TR-FRET) between two anti-$(His)_6$ antibodies, labeled with either a donor or acceptor moiety, which simultaneously bound to the aggregated protein, thus allowing one to monitor the progressive solvent exposure of these affinity tails. The method was demonstrated in both temperature ramp-up mode, as well as isothermal regime; however, weak points of the approach, including cost of TR-FRET reagents, the need to optimize the temperature for each protein, the complications arising from the fact that TR-FRET signal depends on the exact number and spatial orientation of hexahistidine tails along the aggregates' surface, as well as on the ratio of donor and acceptor antibodies populating that surface, have resulted in practically no reported follow-up uses of ATLAS.

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In a very recent example [23], the process of protein unfolding in the presence of chemical denaturants as a function of time was exploited through a modified DSF protocol that incorporated real-time kinetic observation of fluorescence change upon mixing a protein with a denaturant and/or stabilizer, while the temperature was kept constant and the traditional SYPRO Orange environment-sensitive fluorescence dye was used to generate signal. Using hen egg white lysozyme and *S. cerevisiae* hexokinase as test proteins and urea as the denaturant, the authors analyzed the initial linear portions of the fluorescence evolution curves acquired shortly after mixing the temperature-equilibrated protein, dye, and denaturant stocks: half-maximal rates of protein denaturation, as well as the degree of denaturant cooperation, were derived. It should be noted that the utility of this method may be limited primarily due to complications arising from the admixing of protein denaturation rates and the rate of fluorescent dye binding.

Another emerging application of DSF is in the area of discovery of ligands that target nucleic acid structures. This is an exciting area that offers a new avenue for drug discovery as aberrant structures, such as quadruplexes, loops, and branches have been implicated in disease processes. An interesting methodological difference between DSF as applied to nucleic acids is that it typically uses FRET instead of the less specific dye binding phenomenon exploited in proteins, an approach which allows most of the nucleic acid target structure to remain intact and available for the small molecule to bind. Thus, FAM/TAMRA FRET pair has been utilized in T_m measurement protocols in search of binders of various structural motifs, including in the context of high-throughput screening [24–27].

2.2 Ways to use the thermal denaturation data

On a very basic level, higher T_m obtained from a DSF experiment has been associated with increased stability of the protein, presumably through improved folding brought about by interaction with a specific small molecule ligand or through more favorable environment of ionic strength, counterions, and others, a reasoning that follows from similar analyses using differential scanning calorimetry [28]. As such, the immediate application of DSF data has been in the areas of identification of optimal buffer conditions for protein storage or X-ray crystallography, or for rapid stability ranking of protein point mutants or quality of enzyme preparations, including degree of protein degradation [13,29–32]. A relatively large profiling study sought to determine whether the quality of enzyme preparations could be estimated from the corresponding DSF data: 31 recombinant enzymes from *Plasmodium* parasites were profiled using DSF and results were compared with standard enzyme activity assays [13]. In general, the quality of the melting profile and the T_m was correlated with enzyme activity, and the study authors concluded that DSF could be used to separate protein stocks into properly-folded and possibly-denatured categories, thus facilitating downstream work.

Arguably the most ubiquitous use of DSF has been to assess the binding of small molecule drug candidates to their corresponding protein targets, either in HTS discovery mode or as part of the hit validation and characterization process [15]. Compounds that bind with higher affinity are generally thought to shift T_m to a greater extent and there have been favorable comparison studies indicating strong linear correlation between DSF-derived T_m values with those obtained from DSC [33,34]. It is important to note that the shift in T_m has a simple

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thermodynamic meaning only when the receptor is saturated [35]. At less than saturating ligand concentration, melting can in fact appear to be multiphasic due to ligand redistribution, posing challenges in case where T_m determination is performed in an automated fashion through the use of instrument software. Thus, determination of the "true" T_m of a ligand-bound protein can only be accomplished through a concentration-response type of experiment in order to ensure that the binding site has indeed been saturated. In addition to stabilization of a protein by a small molecule ligand, the stabilization of a given protein by another protein, i.e., the formation of a protein-protein complex, was recently reported to be detectable through a careful analysis of DSF melting curves [32].

Thermodynamics analyses of DSC and DSF data, specifically the derivation of binding and energetic parameters such as K_d, ΔH, ΔS, ΔG, have been reported in multiple primary publications and reviews [15,36–38] and as such will not be discussed here in detail. It is important, however, to point out the caveats and potential false interpretations associated with these analyses. While higher binding affinity is ideally accompanied by a greater increase in T_m, multiple factors can conspire to break or even invert this relationship. As detailed in excellent reviews by Zhang, Matulis, Garbett, Holdgate, and others [2,5,29,39,40], the net effect of the interplay between entropic and enthalpic contributions to the binding free energy, as well as changes in heat capacity (sometimes collectively referred to as enthalpy-entropy compensation) is effectively impossible to predict. One manifestation of confusing data trend is that the magnitude of the T_m shift observed for different test compounds with the same affinity at the relevant temperature is dependent on the contributions of enthalpy and entropy to binding and as such larger T_m shifts tend to be observed for more entropically driven (e.g. hydrophobic) binding events. Conversely, identical T_m shifts (for two or more ligands) cannot be uniquely ascribed to one specific binding affinity because a range of different affinities, each with different entropic and enthalpic components, can easily produce the same change in T_m. In addition to enthalpic-*versus*-entropic interplay, a ligand's overall effect may be masked by binding to both the native and the denatured protein states, with a net result being only a minor shift in the equilibrium. In addition to these fundamental issues with data interpretation, more mundane factors, such as running a DSF study using an excess of dye (relative to the maximum hydrophobic binding surfaces that would be exposed upon complete sample melting) can turn the thermostability profiling into essentially a displacement assay with the small molecule binder competing with dye for binding to protein surfaces and thus providing an obscured picture of the overall stabilization process [2].

Two other noteworthy complications are high starting fluorescence (i.e., high fluorescence of the room-temperature sample) and negative thermal stabilization (i.e., decrease in T_m in the presence of a small molecule candidate binder). While the former is almost universally explained as a hallmark of an already-degraded or denatured protein (see detailed study of a V75H$^+$I155A DHFR mutant by Bershtein et al. [34]), the latter can result from a multiplicity of factors. Some authors have associated a decrease of T_m with the small molecule binding more strongly to the unfolded protein [2,3,28,41]. Additionally, destabilization has been attributed to phenomena such as the ligands acting to effect covalent modification of the protein, change in ionic strength leading to a depletion of ions that stabilize the protein, or detergent-like denaturation [42]. Because of the complications surrounding the interpretation

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of a decreased T_m, destabilizers have been largely dismissed and removed from detailed investigations [29]; however, there seem to be no detailed studies on whether all destabilizers can be summarily placed into the nonspecific binder category.

3. Examples of recent DSF use

There has been a significant diversification of DSF applications beyond initial small molecule discovery into areas such as protein therapeutic development, formulation studies, detailed mechanism of action (MOA) studies involving small molecule inhibitors in the backdrop of complex enzymatic reactions, and others. These are described below and summarized in Table 2.

3.1 Protein therapeutics

There has been a rapid adoption of DSF to support the development of protein therapeutics, with the two main areas of application being the identification of most thermostable protein variants (out of a pool of candidate mutants, for example) and the identification of best conditions for storing the protein. An example of the use of DSF to identify the protein variants best fit for further development is provided through the study of Lavinder *et al.* termed "high-throughput thermal scanning" by the authors. In it, a library of protein variants of the four-helix bundle protein Rop was tested under the same conditions of dye and buffer to probe the approximate relative thermal stabilities of the mutants to find the mutations that confer higher stability [30].

DSF is finding an increased use for the development of monoclonal antibody formulations. By comparing the data from accelerated stress tests, Goldberg *et al.* concluded that DSF could be used as a high-throughput method to screen for conformational and colloidal stability during formulation optimization [43]. In a related study, Li *et al.* applied DSF to test the aggregation propensity of three monoclonals and found that T_m alone was not able to predict aggregation pathways, but played an essential role when used in combination with a size based assay to understand the aggregation behavior of the protein [44]. Additional reports cover similar uses of DSF in screening panels of monoclonals and/or formulation conditions [45,46]. It should be noted, however, that good thermal stability, as determined by the heating of a protein sample and measurement of a superior T_m, does not exactly translate into improved resistance of the protein preparation to proteolytic or other type degradation upon prolonged storage at room temperature or at 4 °C.

Most DSF studies in protein formulation development have traditionally used SYPRO Orange as the fluorescent dye. However, despite the wide acceptance of SYPRO Orange, its physical properties preclude the use in DSF studies where surface-active excipients are included, although surfactants are often used as stabilizing agents during production and storage of therapeutic proteins. In the presence of a surfactant above the critical micelle concentration, SYPRO Orange is sequestered into the hydrophobic micelle core resulting in high fluorescence background; in turn, the comparatively small increase of the fluorescence intensity due to the unfolding of the protein is concealed under these conditions.

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To overcome this major limitation in DSF as high-throughput technique in formulation development, a solution has been investigated through the use of a molecular rotor type of probe, 4-(dicyanovinyl)julolidine (DCVJ), that shows environmentally sensitive fluorescent properties [19]. DCVJ's fluorescence was shown to become stronger in the more ordered environment provided by the exposed protein hydrophobic regions, with a comparatively small interference from the micelle environment. Structurally and spectroscopically, DCVJ belongs to the category of TICT compounds: twisted intramolecular charge transfer (TICT) state is reached by intramolecular charge transfer in an electron donor (D)/acceptor (A) portions of a molecule, whereby it is possible under certain conditions of constrained intramolecular group movement for the D and A moieties to adopt a mutually perpendicular configuration that leads to electronic decoupling of D and A and minimization of the nonradiative relaxation path (also referred to as torsional rearrangement) after photon excitation [47]. Although limited to higher antibody concentrations, DCVJ, after a background correction, produced enough signal which enabled the determination of T_m in many formulations where SYPRO Orange had failed, with trends reported to be in good agreement with differential scanning calorimetry measurements [19]. Using this specific dye it now appears feasible to use DSF to guide the development of surfactant-containing therapeutic protein formulations.

3.2 High-throughput screening (HTS) and large-scale profiling of inhibitors and chemical chaperones

The use of DSF to either conduct HTS campaigns for inhibitor discovery or to serve as a secondary validation assay has become widespread. Examples include the use of DSF in direct HTS to discover inhibitors of DOT1L histone methyltransferase ([48], also see below), monoglyceride lipase with a Thermofluor primary HTS followed by a kinetic fluorescent assay using coumarin-based substrate [9], and in an early example the discovery of HDM2 stabilizers through direct DSF HTS [49]. Alternatively, reverse or combined approaches have been reported, such as performing a virtual followed by DSF [50], running a coupled DSF and activity screen in cases where the enzymatic activity assay is exceedingly complicated [11], a fluorescence polarization based HTS followed by DSF for rapid triage in the discovery of HIV-1 NC inhibitors [17], or discovery of small-molecule inhibitor of mixed-lineage leukemia protein complex activity by disruption of its interaction with WD40 repeat protein 5 [51].

A combination approach of thermal stability HTS and rational design resulted in the discovery of a potent inhibitor of DOT1L histone methyltransferase. Noting the commonality in adenosine scaffold shared by the cofactors of methyltransferases and kinases (S-adenosylmethionine (SAM) and adenosine triphosphate (ATP), respectively), a team from the Structural Genomics Consortium screened a library of 3120 kinase inhibitors using DSF to find novel SAM-competitive inhibitors of the histone methyltransferase DOT1L1, a key emerging epigenetics target [48]. 5-iodotubercidine (5ITC) was identified as a potential inhibitor with a modest stabilization of 2.5 °C at 50 μM and an enzymatic IC_{50} value of 18.2 μM. Based on the chemical similarity between the cofactors and 5ITC, the team hypothesized that extending the 5ITC molecule by grafting a portion of the S-adenosylhomocysteine product would result in a superior inhibitor through enabling the

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ligand to occupy a larger fraction of the substrate and cofactor binding pockets of DOT1L. Indeed, the new molecule dubbed BrSAH displayed several hundredfold increase in potency, at an IC_{50} value of 77 nM.

In addition to HTS campaigns directed at discovery of new chemical matter and frequently employing a single protein target at a time, DSF has been used in broad profiling efforts where a set of small molecule ligands is tested against a series of proteins, often belonging to the same gene family. Early example is provided through the pioneering work of the Structural Genomics Consortium [8]: 221 different proteins were profiled against small molecule libraries comprising salts and buffer components, to evaluate conditions for highest protein stability, as well as candidate binders of the active sites of the proteins. In 20 cases, including 9 unique human protein kinases, novel small molecule stabilizers were found, paving the way towards deorphanizing these targets.

Recently, a large profile of poly-ADP-ribose polymerases (PARP), as well as the PARP-related tankyrases, was reported by a group led by the Structural Genomics Consortium, Stockholm. Walberg *et al.* evaluated a series of 185 inhibitors, including tool compounds and agents being tested clinically, for the ability to bind to the catalytic domains of 13 of the 17 human PARP family members including the tankyrases TNKS1 and TNKS2 [52]. The authors first validated the use of DSF through a comparison pilot study using DSF and surface plasmon resonance against a subset of compounds, finding good correlation between the data from both methods. The study was then expanded and showed that some of the most widely used inhibitors, including TIQ-A, 6(5H)-phenanthridinone, olaparib, ABT-888 and rucaparib, bound to multiple PARP family members, suggesting that these molecules are broad-acting. This first-in-kind profiling of PARP family crossreactivities of PARP inhibitors has been made public as a Resource publication [52] and should thus facilitate the interpretation of biological effects of inhibitors and potentially the results from clinical trials, and may provide key information to aid the rational development of agents with improved selectivity profiles.

In a major development within the DSF field, a large scale HTS dataset has been deposited in its entirety by the Broad Institute using the PubChem database [53]. The HTS sought to identify inhibitors of FGF22-mediated excitatory synaptogenesis through discovery of small molecule binders to FGF22. The HTS was run on Roche LightCycler 480 within the 25°C–85°C range, at a 3.6 °C /min ramp rate. A total of 339,623 compounds from the NIH Molecular Libraries Small Molecule Repository were tested at 10 μM final concentration using 384-well plates. Data were uploaded into PubChem as normalized responses, representing what appears to be the largest single-target DSF screening dataset made publicly available to date (PubChem Assay Identifier 651658 [53]). A relatively large number of actives (5,128 reported hits or 1.5% hit rate) were identified from this screen necessitating a detailed hit validation through orthogonal assays.

Another area of drug discovery where DSF is also finding an increased application is the identification and validation of small molecule stabilizers of mutated proteins, referred to as correctors or chemical chaperones, or pharmacological chaperones. The general concept of chemical chaperones has received an increased attention lately, primarily as it relates to

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discovery of small molecules that bind to the mutant form of the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis and by helping the protein fold and get trafficked properly, they act as correctors of the phenotype [54], and in the area of lysosomal storage disorders where chemical chaperones serve to help mutant glucocerebrosidase and related enzymes fold properly and get transported across appropriate cellular compartments to ultimately exert their glycolytic degradation activity [55]. In cystic fibrosis, RDR1 is a CFTR corrector compound first identified in a HTS using isolated nucleotide-binding domain 1 (NBD1) of CFTR which binds directly to isolated NBD1, and is a mild potentiator of CFTR channel activity [56]. DSF was used to demonstrate that RDR1 improves the thermostability of disease mutant ΔF508-NBD1, with further validation of corrector activity in cell surface expression assays [54,56]. The fact that RDR1 displays only a modest corrector activity underscores the challenges for development of correctors solely based on finding stabilizers of a singular protein target. Detailed studies have highlighted the complexity of CFTR structure and activity, as an example of cooperatively folding multidomain membrane protein, by showing that the ΔF508 mutation destabilizes NBD1 both thermodynamically and kinetically, and that correction of either defect alone is insufficient to restore ΔF508 CFTR biogenesis [18], which may explain the limited success of ΔF508 CFTR corrector molecules.

A related emerging area is the discovery of small molecule chemical chaperones for lysosomal storage disorders [55]. These small molecules bind and stabilize mutant lysosomal enzymes, a step which in turn assists with their proper cellular translocation; select chemical chaperones have been shown to be effective in preclinical models and clinical studies of diseases such as Gaucher's and Fabry [55,57]. Discovery and, importantly, chemical optimization of new chemical chaperones for lysosomal storage disorders through the use of DSF is of particular relevance because of the potential superiority of non-inhibitory chaperones, which by definition would be impossible to discover and further optimize through an enzymatic activity type of assay. Non-inhibitory chaperones are expected to be more effective because in order for the rescued protein to perform its catalytic function, there would not be a need for the chaperone to be diluted out and displaced by the incoming substrate as would be the case with the inhibitory PCs. Recent examples of chemical chaperones characterized through DSF include the quinazoline chemical series acting as glucocerebrosidase inhibitors [58]. The discovery of quinazolines prompted further investigations which ultimately resulted in the discovery of pyrazolopyrimidines as an example of noninhibitory chaperones acting to stabilize glucocerebrosidase without occupying the enzyme active site [59].

3.3 General mechanistic investigations

General mechanistic investigations using DSF include a considerable diversity of studies ranging from protein-protein interactions (PPI) to metal stabilization of various proteins, to investigations of cofactor requirements for newly-discovered enzymes. Layton *et al.* demonstrated that PPIs could be measured by analysis of binding-induced shifts in multiprotein thermal transitions. The interaction between *E. coli* maltose-binding protein and the synthetic ankyrin-repeat protein Off730 was analyzed through DSF as a test case revealing that binding between these two proteins could be detected through thermal

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stability shift and quantified by a thermodynamic analysis. Further, interface mutants were used to demonstrate that a range of affinities could be assessed through this approach [36].

An interesting example of using DSF is its application by Herzog *et al.* to probe the suitability of a *Drosophila* protein to serve as a surrogate model for its human ortholog. The transcription factor p53 acts as a tumor suppressor and in a large number of human cancers it is inactivated through mutations in its DNA-binding domain. The *Drosophila* Dmp53 has similar apoptotic functions as its human homolog and is therefore being considered as a convenient model system for studying human cancer mechanisms. The effect of point mutations in Dmp53, corresponding to cancer hot spot mutations in the human protein, on the stability and DNA binding affinity was tested through DSF [16]. The Hp53 and Dmp53 proteins had similar melting temperatures and showed similar energetic and functional responses to cancer-associated mutations, indicating general suitability of using Dmp53 as a model system for studying p53 function and discovery of p53-targeting drugs despite the low sequence similarity between the two orthologues.

DSF has been used to profile the stabilization of proteins by metal ions. McDevitt studied bacterial susceptibility to zinc by performing thermal stabilization measurements of PsaA, the solute-binding protein of a manganese-specific ABC permease encoded by the psaBCA locus [60]. DSF of PsaA in the presence of zinc or the essential metal manganese demonstrated that, although Mn was the native high-affinity cofactor for PsaA, Zn^{2+} could also bind, even though at a lower affinity and that, crucially, Zn-PsaA complex was significantly more thermally stable than Mn-PsaA, suggesting that Zn^{2+} binding might be irreversible. The study concluded that zinc exerts its toxic effect on bacteria by competition for Mn^{2+} acquisition leading to intracellular Mn^{2+} starvation [60].

Another study of metal stabilization focused on the von Willebrand factor (VWF) whose multimers mediate primary adhesion and aggregation of platelets. VWF activity depends on multimer size, which is regulated by a feedback mechanism involving shear-induced unfolding of the VWF-A2 domain and cleavage by the metalloprotease ADAMTS-13 [61]; unfolding of A2 domain requires higher forces when calcium is present. Comparison of the difference in T_m for individual VWF-A domains with native tandem constructs determined in the presence and absence of calcium showed that a significant T_m change is only observed when A2 was part of the construct and that sensitivity to protease degradation of A2 correlated with thermal stability; furthermore, through a comparative metal-ion DSF study, it was shown that the thermal stabilization of A2 was specific for calcium [61]. The study thus highlighted the relevance of metal coordination for mechanical properties of a protein involved in mechanosensing.

Native stabilization of proteins can be promoted not only through metal coordination but also by cognate interaction with their respective cofactors that are required for catalytic function. Buysschaert employed thermal denaturation assays using Thermofluor protocol to probe cofactor preferences of a new protein subfamily: SDRvv, an atypical short-chain dehydrogenases/reductase (SDR) from *Vibrio vulnificus* was found to be devoid of the catalytic tetrad Asn-Ser-Tyr-Lys typically encountered in SDRs [62]. DSF profiles of the enzyme with various cofactors showed that only the 2′-phosphorylated cofactors increased

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the melting temperature of the apo-form. NADPH produced the largest T_m increase, suggesting that it was preferred over NADP, while neither NADH nor NAD^+ produced appreciable stabilizations. The preference for NADPH as a cofactor was further confirmed by ITC and crystallography analyses [62].

3.4 Mechanisms of Small Molecule Inhibition

In recent years, there have been studies of increased sophistication to probe compound mechanism of action (MOA) in systems of growing complexity. As noted in earlier reviews [2,5], studies utilizing co-dependency matrices of compound tested in combination with another binder of the protein (a known inhibitor, a substrate/product, a cofactor) should be expected to provide valuable mechanistic insights. The below studies, where co-dependency tests frequently utilize ranges of compound concentrations, highlight the wealth of information which DSF has provided recently.

15-PGDH catalyzes the inactivation of a number of bioactive prostaglandins, leukotrienes, and hydroxyeicosatetraenoic acids, a function that makes it an attractive target for mechanistic studies and therapeutic interventions in inflammation and cancer. A screen of ~160,000 compounds in a concentration-response format identified novel inhibitors of 15-PGDH that act as noncompetitive inhibitors (denoted compounds 13 and 72) as well as a competitive inhibitor (denoted compound 61), with nanomolar affinity [63]. The 15-PGDH reaction follows several steps of engagement and disengagement of substrate, cofactors, and products, generally described as an ordered bi-bi mechanism. Thus, to investigate the MOA of the new inhibitors, a combination study utilizing DSF, enzymology, and molecular docking was undertaken. In inhibitor-cofactor co-dependency stabilization studies, compound 61 produced T_m shifts of 12.2°C and 2.9°C with NAD^+ and NADH, respectively, indicating a strong preference for 61 in stabilizing 15-PGDH complexed with NAD^+ as compared with NADH, consistent with binding of 61 along the reaction coordinate of 15-PGDH is at the step of complex of 15-PGDH•NAD^+. In turn, compound 13 produced T_m shifts of 7.3°C and 13.5°C with NAD^+ and NADH, respectively, while compound 72, belonging to a different cluster, showed a similar profile with a significantly larger T_m shift in the presence of NADH. Docking experiments, in combination with enzymology and the above DSF profile, yielded a model compounds 13 and 72 bind at step 4 along the 15-PGDH reaction coordinate, mimicking the product and favoring co-complex formation with NADH.

In another recent substrate/cofactor co-dependency study, human UDP-glucose 6-dehydrogenase (hUGDH), a potential target for cancer therapy, was tested for thermal stability in the presence or absence of its substrate/product UDP-glucose/UDP-glucoronate and cofactors NAD^+/NADH. Addition of NAD^+ to the apoenzyme failed to produce a heat signal, suggesting that NAD^+ does not bind to hUGDH unless the enzyme-substrate complex has formed. Substrate (or product) and coenzyme acted in synergy to elicit an increase in T_m, with reported increases being as large as 18 and 14 °C for the UDP-Glc/NADH or UDP-GlcUA/NAD^+ test cases. The enzyme catalyzes, in two NAD-dependent steps without release of intermediate aldehyde, the oxidation of UDP-glucose (UDP-Glc) to UDP-glucoronate (UDP-GlcUA) and the above DSF information, in combination with

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crystallography data, was used to build a model for the mechanism of this complex enzymatic reaction [12].

Park *et al.* used DSF to map out domain flexibility of human farnesyl pyrophosphate synthase (FPPS), whose inhibition provides a therapeutic approach for the treatment of bone-resorption disorders [64]. Inhibition of human FPPS by bisphosphonates is thought to involve closing of the enzyme's C-terminal tail induced by the binding of the second substrate isopentenyl pyrophosphate (IPP), making biophysical profiling of these processes an important tool in the further development of therapeutics. Binding of inorganic phosphate (P_i) to the complex of FPPS and a bisphosphonate inhibitor YS0470 was studied by ITC, but binding parameters could not be derived due to a low heat signal produced. DSF experiments were then performed, with results corroborating the notion that no significant conformational change was induced by P_i binding, confirming that P_i binding does not induce the tail ordering [64]. T_m of human FPPS was found to increase by ~10°C in the presence of YS0470, indicating that the enzyme is more thermally stable in its partially closed state than in the open state. Addition of the secondary ligands PP_i and IPP was found to further stabilize the enzyme, providing insights into development of optimized inhibitors.

While cofactor/co-substrate dependency analyses can provide indication with respect to site of inhibitor binding, excessively high increases in T_m by certain inhibitor types in the presence of a co-substrate can be a sign of a formation of a covalent adduct which spans an extended binding pocket and thus provides an exceptional stabilization of the protein target. Such was the result obtained by Auld *et al.* in a study of unusually potent inhibitors of firefly luciferase (FLuc) [65]. Previous work by the team had established that inhibitors of FLuc can bind to trace levels of enzyme present in the cell lines that incorporate luciferase reporter gene cassettes, stabilize the reporter and thus increase its intracellular concentration (through the protection from proteolytic degradation), ultimately producing the counterintuitive effect of reporter gene activation [66,67]. While several chemotypes proved capable of acting as FLuc inhibitors and intracellular stabilizers, a group of 3,5-diaryl oxadiazoles exhibited a particularly strong potency, with the best members being single-digit nanomolar. In a detailed DSF study that included testing the inhibitors in a dose-response manner in the absence and presence of the FLuc substrate ATP, it was noted that only members of the series that contained a carboxylate in a meta- position were capable of strong stabilization [65]. Subsequently, LC-MS studies, as well as X-ray crystallography, revealed the formation of covalent PTC-AMP adduct within the FLuc active site, thus explaining the extraordinary stabilization of FLuc by PTC124 and the associated paradoxical effect of that molecule in cell-based assays [65].

Recently, the ability of DSF to report on inhibitor mode of action was evaluated using glutathione S-transferase (GST) as a model enzyme that utilizes two substrates and is known to be subject to several distinct types of inhibition [68]. GSTs contribute to the phase II biotransformation of xenobiotics, including toxic chemicals, in a variety of organisms, through catalyzing the conjugation of a variety of electrophiles with glutathione (GSH). GSH analogs and mimetics can compete with both GSH and hydrophobic substrates by occupying both the G-site and the H-site of GSTs, while some electrophilic substrates bind in the hydrophobic region of the H-site and form tight complexes with GST through the

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formation of adducts with the GSH co-substrate. A third category of inhibitors act by noncompetitive mechanism with respect to both GSH and electrophilic substrate, and they are believed to partially occupy either the H-site or the intersubunit cleft of the GST dimer.

Traditional enzymological studies have been difficult to run with GSTs because of the insensitive nature of the activity assay for this enzyme class. Using model inhibitors representing the above three mechanisms, a detailed DSF study where inhibitors were tested against GST isozymes in dose response in the absence and presence of GSH co-substrate revealed distinct thermal shift signatures: GSH-competitive inhibitors produced dose-dependent thermal shift trendlines that converged at high compound concentrations (implying equivalency of stabilization by inhibitor *versus* GSH at high concentrations of both agents), inhibitors acting *via* the formation of glutathione conjugates induced a very pronounced stabilizing effect toward the protein only when GSH was present, while noncompetitive inhibitors exhibited parallel concentration-dependent trends (implying additivity of the individual stabilizing contributions) [68]. The study demonstrated that DSF can provide complementary information on protein-ligand binding pathways using very simple experimental setup which allows the rapid profiling of multiple inhibitors; this tool should be of particular utility in situations where detailed enzyme kinetic studies are difficult to perform.

4.0 Expert opinion

DSF has enjoyed a steadily increasing adoption during the past several years. The fact that a large number of vendors are now providing dedicated DSF kits, instrument protocols, and T_m analysis tools, can be viewed as another sign of increased demand. While recently there have been publications on variations of the technique, the vast majority of studies continue to follow the initially-introduced protocol. There may be at least two reasons for such an adherence: on the one hand, isothermal, kinetic, and related approaches have not been convincingly demonstrated to provide superior datasets compared with the traditional temperature ramp-up regime; on the other hand, the modified protocols generally appear to be more difficult to set up and validate.

There has been a significant diversification of DSF applications beyond initial small molecule discovery into areas such as protein therapeutic development, formulation studies, and various mechanistic investigations, serving as a further indication of the broad penetration of the technique. In the protein formulations area, a welcome development has been the recent identification of an alternative reporter dye to enable DSF deployment for samples containing detergents where SYPRO Orange has tended to fail. In the small molecule arena, DSF use has not only continued the early trend of providing complementary evidence of compound binding [5] but has expanded into sophisticated co-dependency MOA tests, demonstrating the wealth of information which the technique can provide. The significant advances in small molecule MOA studies are in part due to the increased testing of ligands in dose-response format. Concentration-response testing is important not only because a dose-range data allow for a better evaluation of the actual interaction trends, but also because single-concentration tests carry the risk of generating erroneous data in the cases where the compound happens to be insoluble at the concentration applied [2]. Lastly, a

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welcome development in small molecule DSF is the first public deposition of a large screening dataset, a step which will hopefully enable the use of thermal stabilization data in refining *in silico* models for small molecule binding. As efforts to illuminate the druggable genome intensify, HTS by DSF is expected to become an even more important tool to discover selective ligands of proteins for which functional assays have not yet been configured. As related to these efforts, it is important to note that while DSF data acquisition is gaining in throughput, data analysis has definitely lagged behind, with human intervention often required to inspect the large number of melting curves generated, making enhancements of the analysis software especially necessary.

In closing, widespread use of DSF is expected to continue, with utility expanding beyond proof of stabilization of protein targets by small molecules to using the technique to inform on MOA in multisubstrate reactions and multipartner binding events. While competing techniques continue to emerge, as in the example of array calorimetry [69], the simplicity of DSF remains hard to surpass. Going forward, it is hoped that technological improvements will allow for further miniaturization so that additional protein savings are realized and larger screens enabled. Of note, a 1536-well plate based real-time thermocycler already exists, potentially opening the door to running DSF experiments in low-volume settings, with adjustments needing to be made mostly to the software in order to run and analyze high-resolution thermal melts.

List of abbreviations

DSF	differential scanning fluorometry
IC_{50}	concentration that produces 50% inhibition
ANS	anilinonaphthalenesulfonic acid
HTS	high-throughput screening
PubChem AID	PubChem Assay Identifier
MOA	mechanism of action
SAR	structure-activity relationship
PPI	protein-protein interactions

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Highlights

- Differential scanning fluorometry (DSF) is a convenient method to evaluate the thermal stability of proteins under a range of conditions.
- DSF has enjoyed a steadily increasing adoption during the past several years along with a dramatic increase in number of vendors providing dedicated DSF kits, instrument protocols, and T_m analysis tools.
- Adoption of DSF is being noted in the area of protein therapeutic development.
- Very large HTS datasets being published, for example, the dataset from a ~340,000-compound HTS campaign at the Broad Institute deposited into PubChem.
- Evolving trends are discussed, specifically the use of DSF in increasingly sophisticated mechanism of action (MOA) studies in cases of enzymatic reactions involving multiple substrates.

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Table 1

Variations of the DSF technique.

Name	Brief description	Reported or intended application	References
Thermofluor	Initial introduction of the thermal stabilization technique for high-throughput applications	Screening platform for small molecule stabilizers of protein targets	[10]
DSF/thermal shift	T_m measurement technique now commonly used with real-time thermocyclers and SYPRO Orange as detector dye; additional dyes being introduced	Discovery of stabilizing ligands, evaluation of storage conditions, stability evaluation of protein variants, mechanism of action of small molecule inhibitors, selectivity profiling	[1–5,7,19,36,45,52,68,70]
Isothermal	Kinetic detection of fluorescence evolution upon heating the samples to approx. 4 °C below protein T_m	Simplified HTS protocol to find preferential stabilizers of citrate synthase	[20]
ATLAS	TR-FRET based detection of exposure of protein hexahistidine tails upon protein denaturation; performed as temperature ramp or isothermal	A version of DSF that utilizes a more sensitive detection than afforded by SYPRO Orange	[21,22]
Kinetic DSF	A version of isothermal DSF where fluorescence intensity is monitored in real time after an addition of a chemical denaturant and/or stabilizer	Expected to provide a more detailed view on the kinetic aspects of protein unfolding and the interplay between heat- and chemical-induced denaturation	[23]

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Table 2

Recent applications of DSF.

Test system	Study design	Study purpose, outcome	References
Protein Therapeutics	T_m determination for sets of protein variants or same protein across different buffer or formulation conditions	Selection of best variant for further development, selection of storage conditions, improvement of formulations	[19,30,43–46]
HTS	DSF-driven screens of small molecule libraries followed by orthogonal validation assays, e.g. DoT1L, monoglyceride lipase, HIV-1 NC, MLL/WDR5, FGF22	Discovery of novel ligands for select protein targets, accompanied recently by public deposition of large datasets	[9,17,48,51,53]
Profiling	A set of small molecule ligands tested against a series of typically related proteins, e.g. kinases, PARPs	Provides selectivity/promiscuity profiles of bioactive small molecules to understand off-target effects and improve development	[8,52]
Discovery of Chemical Chaperones	Use of DSF to initially discover and/or to further characterize small molecule chaperones of mutant proteins in rare genetic diseases, e.g. correctors of ΔF508/CFTR, chaperones of glucocerebrosidase	Discovery of novel ligands for select protein targets, particularly relevant in cases where noninhibitory protein stabilizers are desired	[54,56,58]
Stabilization by Cofactors or Other Proteins	Profiling proteins for stabilization by protein binding partners and by combinations of cofactors and/or reaction substrates and products, e.g. PPI between *E. coli* MBP and Off730, *Vibrio* SDRvv cofactor, Ca^{2+} stabilization of vWF	Provides insights into reaction mechanism, mechanisms of folding and stabilization of multidomain proteins, aids in the discovery of native cofactors of new proteins, ascertains formation of protein-protein complexes	[36,60–62]
Mechanism of Action of Inhibitors	Cofactor and substrate co-dependency studies of protein stabilization to identify competitive-*vs*-noncompetitive modes, and to identify unusual modes of stabilization such as adduct formation, e.g. MOA of 15-PGDH inhibitors, PTC124-AMP adduct, GST MOA studies	Provides initial insights into MOA, useful when the assay for the underlying enzymatic reaction is difficult to scale up for full enzyme kinetics study	[63,65,68]