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Research article

Fluorescence-based thermal stability screening is concentration-dependent and varies with protein size

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Abstract

Proteins are used in many areas including diagnostic and therapeutical applications. Screening protein stability is an essential step for production, pharmacokinetic/pharmacodynamic studies, and determination of storage conditions. Instability of proteins can cause serious problems such as activity loss and unexpected adverse effects, so determination of sensitive and reliable methods for protein stability measurement is crucial. There are several "gold-standard" protein stability tests such as differential scanning calorimetry (DSC), but they are usually not suitable for high-throughput settings and consume large amounts of proteins. Instead, more high-throughput methods such as fluorescent-based assays can be used and validated to make stability screening process more straight-forward, easier, and lower-cost. Here, two methods were systemically compared to see whether their measurements depended on protein sizes. DSC and Sypro Orange dye-based fluorescent assay were compared for various proteins with different sizes and quaternary structures. This is the first systemic comparison of these two methods for thermal stability testing for different ranges of proteins in the literature. It was shown that protein melting temperature (T_m) measured by fluorescent assay highly depends on protein concentration and protein size. Larger proteins with multi-domain structures such as monoclonal antibodies gave more deviated and lower than expected T_ms compared to small proteins. It has been concluded that fluorescent-based thermal stability assays are more suitable for smaller proteins, but protein concentrations used are still needed to be optimized in their settings for more reliable results.

Keywords: *Differential scanning calorimetry; fluorescent dye; thermal melt; thermal stability; protein stability*

1. Introduction

Chemical and physical instabilities are challenging for development of protein therapeutics. Chemical instability involves processes that change chemical properties by modifying covalent bonds. Physical instability includes processes such as precipitation, adsorption, denaturation, and aggregation (Manning et al., 1989; Akbarian and Chen, 2022). In the production and development of protein therapeutics, it is essential to screen protein stability during various processes such as expression, purification, formulation, biophysical characterization, *in vivo* testing, and storage. It is important to determine protein stability in the easiest and cheapest way, especially in areas where various biotechnological products such as protein-based therapeutics, reagents, antibodies, and industrial enzymes are developed (Stourac et al., 2021).

Various techniques are being used to determine protein stability, mainly classifying as label-required and label-free methods (Gromiha, 2010; Rufer and Hennig, 2020). There are also various computational approaches to estimate the thermal stability with improved accuracies (Miotto et al., 2019; Jana et al., 2020; Jung et al., 2023). The most common approaches include differential scanning calorimetry (DSC) (Gill et al., 2010), circular dichroism (CD) (Fiedler et al., 2013), fluorescence-based thermal-shift assays (Lavinder et al., 2009), nuclear magnetic resonance (NMR) (Puglisi et al., 2020), pulse-

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chase methods (Elgendy, 2017), light scattering and gel filtration (Al-Ghobashy et al., 2017). Most widely used label-free method is DSC, which is referred as "gold standard" for protein thermal stability analysis (Vuorinen et al., 2020; Kopra et al., 2022). DSC is a thermal analyzer that examines how the physical properties of a sample are altered with temperature depending on time. It is known that conformational states and physicochemical stability of proteins are thermodynamically-driven (Araya et al., 2012). Thus, direct measurements of thermodynamic parameters and heat effects by DSC is convenient to determine protein thermal stability. However, DSC and some of other label-free techniques requires high amount of protein and they do not adapt to high-throughput experimental settings.

Fluorescence-based assays are very convenient methods to observe and examine protein stability through protein thermal shift technique (Engrola et al., 2023). Various extrinsic fluorescent dyes are being used for high-throughput protein characterization (Hawe et al., 2008; Kirley and Norman, 2022). Because these dyes form only non-covalent interactions with the protein and its solvent environment, they are great tools for accurate predictions of protein states. Besides stability testing, these dyes can be used to analyze various properties of proteins such as hydrophobicity (Cardamone and Puri, 1992), unfolding/refolding (Acharya and Rao, 2003), aggregation/fibrillation (Vetri et al., 2007), crystallization (Groves et al., 2007) and chemical degradation (Anraku et al., 2001). The protein thermal shift assay basically involves incubation of a naturally folded protein with a fluorescent dye and analyzing the fluorescence signal by either a fluorometer or more high-throughput settings like real-time PCR instrument (Layton and Hellinga, 2011). Various dyes such as Sypro Orange, 1.8-ANS (1-anilinophthalene-8-sulfonate) and 2.6-TNS (naphthalene-6-sulfonic acid) are among the common dyes used for protein stability testing (Huynh and Partch, 2015). Most of these dyes provide sensitive detection by fluorescence spectroscopy but have excitable wavelengths outside the range of real-time PCR instruments. Sypro Orange is a dye which binds to hydrophobic parts of proteins to exhibit a fluorescent effect. One of the advantages of using Sypro Orange dye in protein thermal shift analysis is that its fluorescent properties are compatible with filter sets found in high-throughput real-time PCR instruments (Tresnak and Hackel; 2023; Warrender et al., 2023).

In this study, the aim was to compare two most used methods, DSC and fluorescence-based assay for protein thermal stability analysis. While DSC is an excellent label-free method, it has disadvantages such as excessive sample consumption, costly equipment, and time-intensive. More high-throughput and less costly methods are desirable, but their sensitivity and reliability should be comparable to a "gold-standard" method such as DSC. Fluorescent thermal shift assay method based on Sypro Orange dye is very advantageous in both academia and industry due to its low sample consumption, high-throughput potential, use of widely common real-time PCR equipment, and time/cost efficiency (Redhead et al., 2017; Kazlauskas et al., 2021). While DSC is a concentration-independent method (Nemergut et al., 2017), fluorescent-based methods can be concentration-dependent which should be analyzed thoroughly for each protein system. Here, the protein- and dye-concentration dependencies and protein size effects on fluorescent-based thermal assays were documented. According to the results of this study, certain ranges of fluorescent dye and protein concentrations affect the obtained melting point results. While 5X Sypro Orange dye concentration are optimum for all proteins, lower protein concentrations (2-8 μM) give the closest T_m results to those of DSC. It can be concluded that dye/protein concentrations in fluorescence-based thermal shift set-ups should be selected based on the size and domain structure of used proteins to obtain reliable thermal stability results.

2. Materials and methods

2.1. Proteins

Receptor-binding domain (RBD) of the SARS-CoV-2 spike protein (amino acids 332-550) was produced *in house* in *Pichia pastoris* system (Kalyoncu et al., 2023). This RBD was a kind gift of Mehmet Inan at Izmir Biomedicine and Genome Center. Commercial bovine serum albumin (BSA, Diagnovum-D661), bevacizumab (Roche), and adalimumab (Abbvie) were used. Concentrations of proteins were measured by their absorbance at 280 nm via NanoDrop (Thermo Fisher).

2.2. Differential Scanning Calorimetry (DSC)

Protein thermal stability and the thermodynamic parameters of protein denaturation were estimated by using a nano-DSC (TA Instruments) equipment. Before DSC measurements, protein solutions (1.2 mg/ml receptor-binding domain (RBD) of the SARS-CoV-2 spike protein, 1 mg/ml bovine serum albumin (BSA), 1 mg/ml bevacizumab, 1 mg/ml adalimumab) were prepared in 1X phosphate-buffered-solution (PBS, pH 7.4) at room temperature. Each sample was placed in a degassing device (TA instruments) at 15 Hg for 15 minutes before measurement. For blank measurement, 1 mL of 1X PBS was loaded into the instrument. The measurements were done in the temperature range of 25-98°C, with a scanning rate of 1°C/min. At least two measurements were done for each protein. The partial heat capacity contribution from 1X PBS and proteins in 1X PBS were measured independently and subtracted. The calorimetric data were corrected for the calorimetric baseline between the initial and the final state by using a sigmoidal baseline from NanoAnalyze software. DSC traces were deconvolved using OriginPro 8.5 software and melting temperatures (T_m) were represented.

2.3. Fluorescence-based thermal stability analysis

Thermal melt profiles of each protein were determined with SYPRO Orange dye (Sigma-S5692) via ABI 7500 Fast RT-PCR instrument. Each protein was diluted in 1X PBS (2, 4, 8, 16, 24, 32 μM), and mixed with SYPRO Orange dye (2X, 5X, 10X) in opaque 96-well PCR plates and sealed with foil. Fluorescence readings were obtained between 25-99°C with 0.05% ramp rate. Three replicates in the plate were done for each protein and each experiment was repeated at least two times at different days. Obtained melt curves were background subtracted using background signals obtained with 1X PBS control. Transition mid-points (T_m values) from the thermogram data were calculated using the Hill1 equation fit using the OriginPro 8.5 software.

3. Results and discussion

The thermal stability of proteins is only partially

understood despite its great importance in both scientific and industrial fields. Adopting suitable methods for thermal stability measurement will undoubtedly contribute greatly to understanding of physicochemical determinants of protein stability. While "gold standard" methods such as DSC are great tools for stability determination, more high-throughput methods are needed to screen more proteins and conditions at a time. Therefore, reliability of these high-throughput methods should be tested for different proteins because protein size and quaternary structure could be important for their sensitivity.

In this study, four different proteins ranging from small, single-domain proteins to large, multi-domain proteins were tested (Fig. 1). Receptor-binding domain (RBD) of SARS-CoV-2 virus which is 218 amino acids long (332-550 of the Spike protein for SARS-CoV-2) were used (Kalyoncu et al., 2023). RBD is single domain protein with a molecular weight of 25 kDa. Bovine serum albumin (BSA) has three domains with a molecular weight of 66 kDa. As multi-domain proteins, two therapeutic monoclonal antibodies were used: bevacizumab and adalimumab. They are traditional Immunoglobulin G type 1

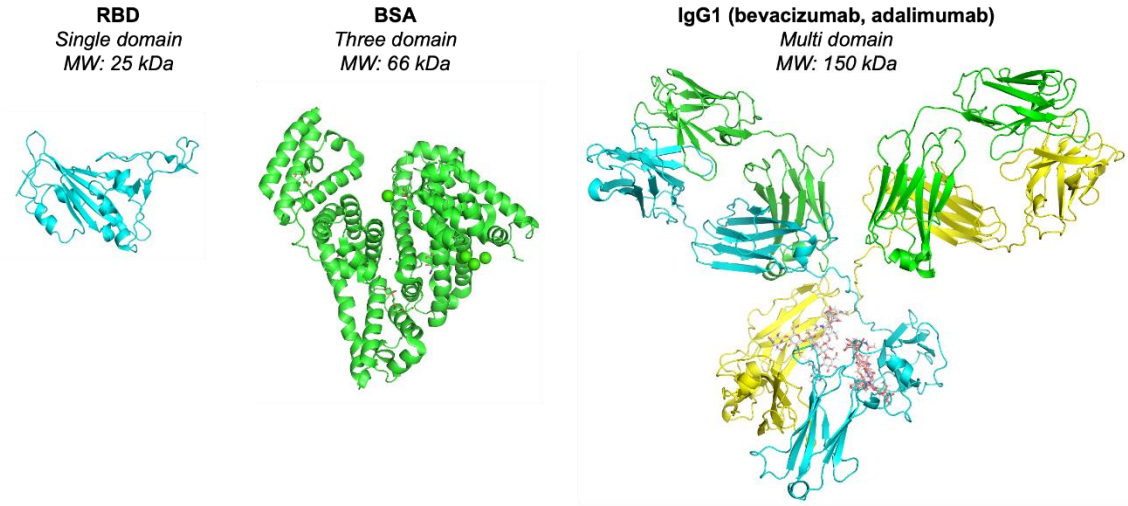


Fig. 1. Structures of proteins used in this study. Cartoon representation of RBD (left, PDB ID: 7E7Y), BSA (middle, PDB ID: 4F5S), IgG1 representing bevacizumab and adalimumab (right, PDB ID: 1HZH). Their domain structures and molecular weights (MWs) are represented on top of each structure.

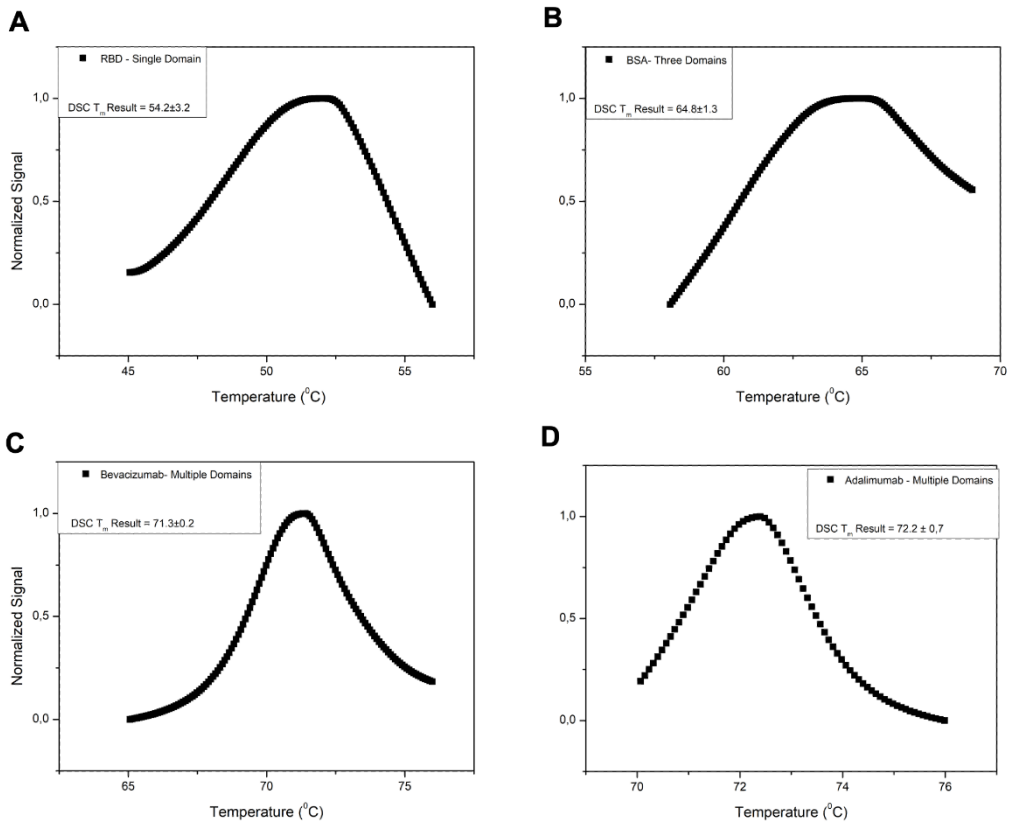
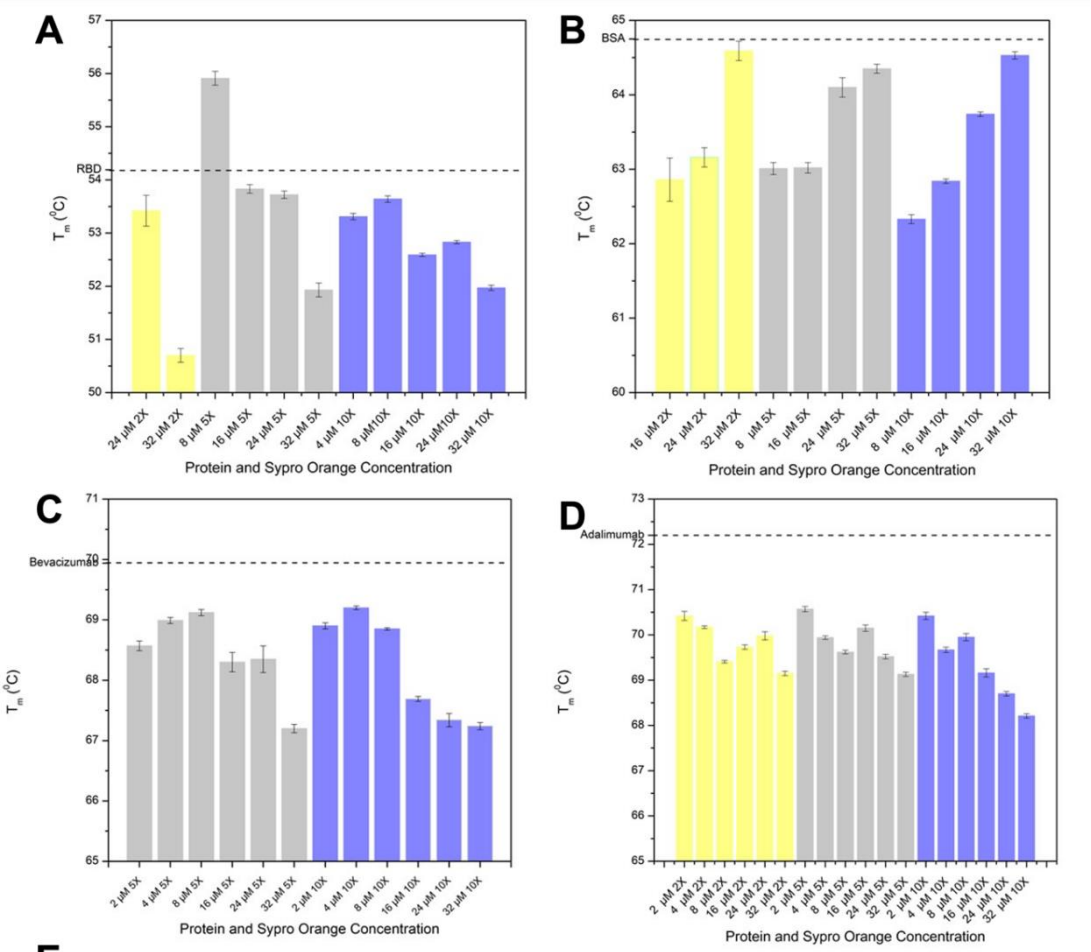


Fig. 2. DSC thermograms of proteins used in this study. Normalized DSC thermograms and calculated thermal melting point (T_m) of (A) RBD (B) BSA, (C) Bevacizumab, (D) Adalimumab.

(IgG1) antibodies with a multi-domain structure and a molecular weight of 150 kDa (Fig. 1).

As a gold standard method (Kopra et al., 2022), DSC was first performed to determine their thermal stability (Fig. 2). While RBD and BSA gave single peak, IgGs gave multiple peaks as expected. The first dominant peak of IgGs which represent Fab region was only potted for comparison (Nemergut et al., 2017), because fluorescent-based methods are usually not that sensitive to resolve multiple transitions. While RBD and BSA gave T_ms of 54.2°C and 64.8°C, respectively (Fig. 2A, 2B), IgGs gave high T_m values around 71-73°C as expected (Fig. 2C, 2D). A large range coverage in T_m values were also targeted from around 50 to 70°C for this study to see whether fluorescent-based methods depend on increasing/decreasing thermal stability.

A high-throughput fluorescent based thermal denaturation experiment was performed in a 96-well setting screening both protein concentration and Sypro Orange dye concentration at the same time in triplicates (Fig. 3). Recommended protein and dye concentrations for this set-up were 2-20 μM and 0.5-10X, respectively (Huynh & Partch, 2015). Proteins were screened in 2-32 μM range and Sypro Orange in 2-10X range. While small proteins gave closer values to DSC T_m values (Fig. 3A, 3B, 3E), large proteins gave more deviated and lower T_m values compared to that of DSC (Fig. 3C, 3D, 3E). In some cases, 2X dye concentration did not gave any reliable data, so they were not included for some of proteins. Also, for RBD and BSA, the lowest protein concentration (2 μM) did not give any reliable signal. This shows that lower Sypro Orange concentrations for all proteins and lower protein concentrations for small proteins are not suitable for this type of assay. Dye concentration of 5X gave the closest T_m to the DSC data for all proteins used (Fig. 3E). Overall, T_m values obtained from fluorescent-based assay were consistently lower than those of "gold standard"s. While smaller proteins (RBD and BSA) gave more accurate T_m values, larger proteins had much lower T_ms. High deviation in multi-domain proteins were expected due to their allosteric effects of each domain on the stability (Oh et al., 2023). Independent of



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		Average T_m from Sypro Orange (°C)								
	T_m from DSC (°C)	2X dye	5X dye	10X dye	2 μM	4 μM	8 μM	16 μM	24 μM	32 μM
Adalimumab	72.2 ± 0.70	69.81 ±0.05	69.82 ±0.07	69.35 ±0.07	70.47 ± 0.08	69.93 ± 0.04	69.66 ± 0.05	69.68 ± 0.07	69.40 ± 0.06	68.83 ± 0.05
Bevacizumab	71.3 ± 0.20	n/a	68.42 ±0.11	68.20 ± 0.05	68.73 ± 0.07	69.10 ± 0.04	68.99 ± 0.04	68.00 ± 0.10	67.85 ± 0.17	67.22 ± 0.07
RBD	54.2 ± 3.20	52.06 ± 0.21	53.85 ±0.10	52.87 ± 0.05	n/a	53.31 ± 0.06	54.78 ± 0.10	53.21 ± 0.06	53.32 ± 0.13	51.53 ± 0.10
BSA	64.8 ± 1.30	63.01 ± 0.04	63.68 ± 0.04	63.56 ± 0.04	n/a	64.35 ± 0.03	63.46 ± 0.05	62.93 ± 0.04	63.21 ± 0.04	63.93 ± 0.04

Fig. 3. Fluorescence-based Sypro Orange thermal melt results. Calculated thermal melting point (T_m) of (A) RBD (B) BSA, (C) Bevacizumab, (D) Adalimumab from Sypro Orange based thermal melt. T_ms were calculated from transition mid-points of Hill equation fittings. Dotted lines in each plot represent "gold-standard" T_m of each respective protein measured by DSC. (E) Comparison of average T_m values from DSC and Sypro Orange based thermal melt. Averages were calculated based on each varying dye and protein concentrations, the values closest to the "gold-standard" T_m obtained from DSC were highlighted.

dye concentrations, lower protein concentrations (2-8 μM) gave T_ms closer to that of their actual T_m (Fig. 3E).

Thermal stability of a protein directly affects its performance. Stability has become a very important issue by researchers and manufacturers, especially with the increasing use of proteins/antibodies in therapeutics, and diagnostics. Instability of proteins has been reported as a major problem in all aspects of research and development (Schuster et al., 2020). Instability in therapeutic proteins can change its pharmacokinetic and pharmacodynamic properties and it can cause adverse effects such as undesired immune response (Boll et al., 2017).

Since protein stability directly affects its production, efficacy, and storage conditions, accurate measurement and screening of their stability is of great importance. High-throughput assays are needed to screen protein stability parameters in various settings, but their sensitivity and reliability should be tested for any protein format. Although it was known that some additives, buffers and concentrations can affect the results of stability analysis (Kopra et al., 2022; Engrola et al., 2023), roles of protein characteristics on the stability measurement are not widely discussed in the literature. Here, a fluorescent-dye based high-throughput assay was compared with a gold standard method.

According to dye-based assays, it was shown that calculated T_ms highly depend on protein concentration and protein size. Larger proteins with multi-domain structures such as monoclonal antibodies gave more deviated and lower T_ms compared to those of small proteins. This means that these fluorescent-based high-throughput assays are more suitable for smaller proteins, but protein concentrations are still needed to be optimized in these settings for more reliable results.

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Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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