Breeze through membrane protein stability with Uncle

Introduction

Membrane proteins are some of the most important and common drug targets in medicine. Mutated membrane proteins cause disease and are biomarkers for cancers, viral and bacterial membrane proteins are important vaccine candidates, and even wild-type membrane proteins may be important for finding cures. Purifying membrane proteins usually involves releasing the protein from the lipid bilayer and coating the hydrophobic regions of the protein with a surfactant to create a detergent-solubilized form. This solubilization often destabilizes membrane proteins, making it difficult to work with them.

Enhancing the stability of membrane proteins can greatly aid in working with them, but simply characterizing their stability poses significant challenges. Established methods, like differential scanning calorimetry and NMR, often require large amounts of protein and are difficult to adapt to higher throughputs. Given the difficulty in purifying large quantities of stable membrane proteins, this puts such assays out of reach. Assaying bioactivity of some membrane proteins, like G-protein coupled receptors (GPCRs), in their detergent-solubilized state can be quite challenging as well. Each of these methods only give a single perspective, resulting in an incomplete picture of the membrane protein's stability.

Uncle is the original all-in-one platform for protein stability that uses 3 detection methods: full-spectrum fluorescence, static light scattering (SLS), and dynamic light scattering (DLS) to fully profile protein stability from 9 µL of sample (Figure 1). Temperature control (15–95 °C) and sealed samples supply greater flexibility in how that profiling can be performed. Multiple parameters, including

Figure 1: Uncle is the original all-in-one biologics stability platform.

fluorescence, aggregation, size, polydispersity, and thermal unfolding, can be assessed from the same sample in just 1 experiment, allowing you to obtain orthogonal and complimentary information on protein stability. Uncle can measure up to 48 samples at a time, enabling greater throughput and broader, faster results when characterizing membrane proteins.

Label-free methods are best for determining protein unfolding, for example using changes to the ultraviolet-induced intrinsic fluorescence of the protein upon melting. However, this method may not be appropriate for every membrane protein as the partially lipophilic environment can make it hard to see the fluorescence shift typically observed in soluble proteins.

The thiol-reactive fluorescent dye 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) can be used to monitor the unfolding of membrane proteins by emitting more strongly after reacting with cysteine residues that are exposed during unfolding caused by a

temperature increase.[1] CPM thermostability assays have also been suggested as alternatives to expensive, time-consuming cell-based assays for drug screening.[2]

Nanodiscs and enveloped virus-like particles (VLPs) are 2 alternatives to detergent solubilization which incorporate membrane proteins into environments that are more similar to the lipid bilayer and may stabilize the proteins.[3,4] Nanodiscs are disc-shaped lipid bilayers which self-assemble from a mixture of detergent-solubilized membrane protein, phospholipid micelles, and membrane scaffold proteins. VLPs also self-assemble and can concentrate membrane protein 'cargo' on the envelope surface before budding from the producing cell. While these types of vehicles for membrane proteins have many advantages, they bring their own challenges to stability characterization which can be solved by Uncle's multi-modal thermal stability approach.

In this application note we show how to use Uncle's full spectrum fluorescence, SLS, DLS, and CPM dye to determine the stability of GPRC5D, a membrane protein biomarker and potential immunotherapy target for multiple myeloma, and CD20, a surface antigen on B cells targeted by Rituximab®, in various carrier vehicles.[5,6]

Methods

Human GPRC5D Detergent, Nanodisc, and VLP (Catalog #s GPD-H52D3, GPD-H52D4, and GPD-H52P5), CD20 Detergent, Nanodisc, and VLP (Catalog #s CD0-H52H3, CD0-H52H1, and CDP-H52P6) and VLP Control (Catalog # VLP-N5213) were generously provided by ACROBiosystems.

GPRC5D and CD20 samples were normalized to approximately 0.15 and 0.25 mg/mL, respectively, in the appropriate buffer. Detergent-solubilized proteins were suspended in 50 mM HEPES, 150 mM NaCl, 0.05% DDM, 0.01% CHS, pH 7.5, with 10% glycerol. GPRC5D Nanodisc was in a formulation with 50 mM HEPES, 150 mM NaCl, pH 7.5, with 20% glycerol. CD20 Nanodisc was in a formulation with 20 mM HEPES, 150 mM NaCl, pH 7.5, with 10% trehalose. All VLPs were in a formulation with phosphate-buffered saline (PBS), pH 7.4, with 10% trehalose.

CPM (Thermo Fisher Scientific) was dissolved to 5 mg/mL in dimethyl sulfoxide, aliquoted, and stored at -80 °C. Immediately before use, an aliquot of the dye stock was thawed at room temperature and diluted to 0.2 mg/mL in the corresponding protein sample formulation buffer. CPM was added to the protein samples to final molarities based on the number of non-disulfide cysteine residues in the protein: 17:1 for GPRC5D and 3:1 for CD20.[7]

Nine microliters of each sample were loaded into cuvettes of a Uni (array of sixteen 9 µL quartz cuvettes, sealed by silicone gaskets) in triplicate for each of the experiments. Samples were ramped in Uncle from 15–95 °C at a rate of 0.6 °C/minute and excited either by 266 and 473 nm lasers together or 266 nm laser alone for CPM-containing samples. The fluorescence emission and SLS were monitored simultaneously.

Uncle Analysis software determined the T_m for samples without CPM from the barycentric mean (BCM) of the fluorescence emission from 300–430 nm. CPM-based T_ms were found from the increase in dye fluorescence from 400–525 nm. T_ms are depicted on fluorescence graphs as vertical lines and are the average of triplicate measurements. The average hydrodynamic diameter before and after the thermal ramp was measured by DLS, with 4 acquisitions of 5 seconds each.

Results

Uncle's 266 nm laser excites tryptophan, tyrosine and phenylalanine amino acid residues in proteins which have a fluorescence peak at approximately 330 nm (Figure 2A). This intrinsic fluorescence usually decreases and shifts towards 350 nm as the protein unfolds and the aromatic amino acids become exposed to a more aqueous environment. Uncle follows the changes in intrinsic fluorescence to track unfolding. If the protein aggregates in the thermal gradient, the intensity of the static light

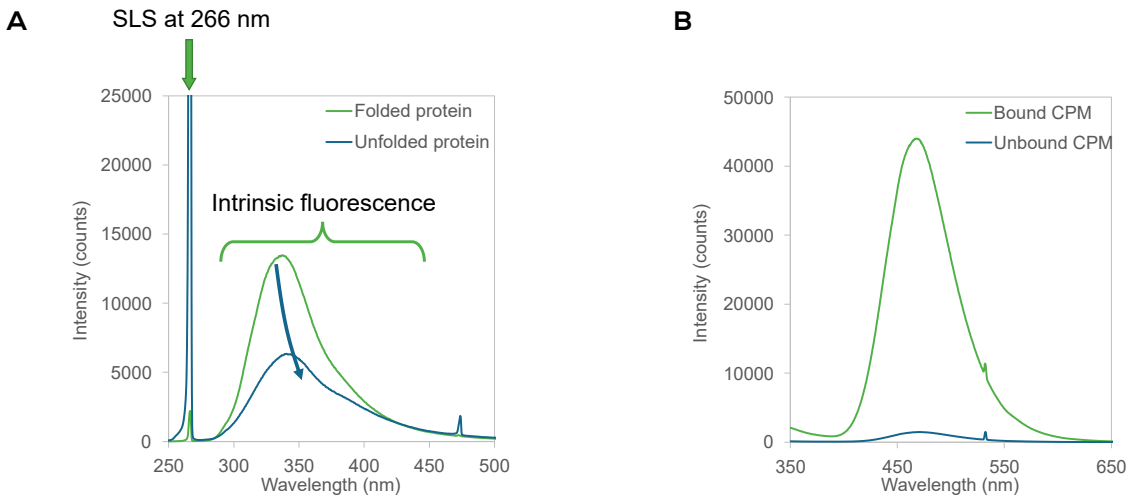

Figure 2: A protein's intrinsic fluorescence tends to decrease and shift to longer wavelengths as it unfolds; as it aggregates, it scatters more light. Uncle simultaneously monitors the increase in SLS and the change in intrinsic fluorescence to assess thermal stability (**A**). The reporter dye CPM reacts with free cysteine residues that become accessible during melting in a thermal ramp. This increases CPM fluorescence, allowing its use in assessing membrane protein stability (**B**).

scattering of the sample increases, which can be monitored along with fluorescence.

Membrane proteins do not always show a change in their intrinsic fluorescence during unfolding due to their solubilization. To allow characterization of their unfolding, Uncle's UV laser can excite CPM, a dye which reacts with the sulfhydryl groups of free cysteine residues of proteins, increasing its fluorescent emission between 400–600 nm (Figure 2B). These cysteine residues become more accessible to the dye as the protein unfolds.

The thermal stability of proteins, even detergent-solubilized membrane proteins, can be ranked based on their unfolding temperature. Uncle's full-spectrum fluorescence detection lets you use different methods to detect unfolding, which gives you the flexibility to find the most appropriate tool for the job. Detergent-solubilized GPRC5D had a CPM T_m of 46.2 °C while its intrinsic fluorescence T_m was 48.8 °C (Figure 3A). The 2.5 °C difference between the T_ms is small and not at all surprising, keeping in mind that the 2 different methods monitor different constituents and possibly domains of the protein.

GPRC5D embedded in nanodiscs had a CPM T_m of 56.6 °C but did not have a discernible

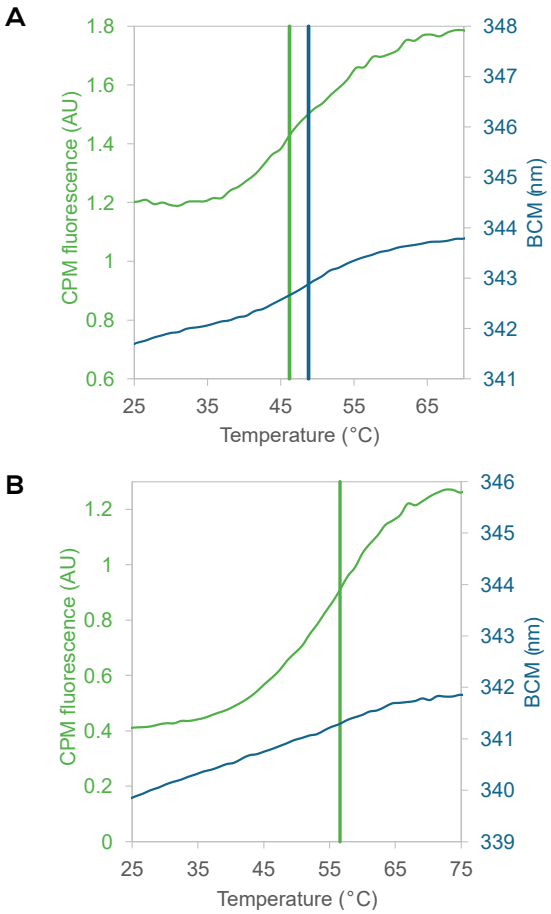

Figure 3: GPRC5D in detergent (**A**) had a single T_m based on the increase in CPM fluorescence (green) and change in intrinsic fluorescence (blue). The CPM T_m of GPRC5D embedded in nanodiscs (**B**) was about 10 °C higher than that observed in detergent. An intrinsic fluorescence T_m could not be assigned to the protein in nanodiscs. Representative fluorescence graphs of triplicates are shown.

intrinsic fluorescence T_m (Figure 3B). The nanodisc scaffold protein MSP1D1 does not have any cysteine residues so the CPM T_m can be attributed to GPRC5D alone. However, MSP1D1 does have aromatic residues that would contribute to the intrinsic fluorescence signal. Since the CPM T_m of GPRC5D in nanodiscs is more than 10 °C higher than that of the detergent-solubilized form, it's likely the nanodisc has a stabilizing effect on the membrane protein.

CD20 has fewer free cysteine residues than GPRC5D (3 vs. 17) and the residues are in either the cytoplasmic or extracellular domains, while GPRC5D also has cysteine residues in its transmembrane alpha-helices. This different arrangement of amino acids may explain why detergent-solubilized CD20 had 2 CPM T_ms (rather than 1 observed in GPRC5D) at 43.2 and 50.9 °C (Figure 4A). While the intrinsic fluorescence emission of CD20 in detergent changed in the thermal gradient, no T_m could be assigned.

Nanodisc-embedded CD20 had 2 CPM T_ms at 44.2 and 51.9 °C (Figure 4B). These T_ms are ~1 °C higher than the detergent T_ms, suggesting that, like GPRC5D, nanodiscs may stabilize CD20. CD20 in nanodiscs had an intrinsic fluorescence T_m at 82.4 °C. No such T_m was present in the GPRC5D nanodisc formulation, indicating the T_m belongs to CD20, not the nanodisc scaffold protein. Uncle's full spectrum fluorescence detection made it possible to more thoroughly investigate the unfolding behavior of the membrane proteins CD20 and GPRC5D in different vehicles.

VLPs are extremely useful vehicles for membrane proteins because the viral envelope is derived from the lipid bilayer of the cell membrane. However, interpreting the intrinsic fluorescence signal of a VLP is complicated by the fact that each viral protein has its own signal that can obscure that of the membrane protein cargo. CPM fluorescence of VLPs has a similar problem. In this case, access to an isotype control allowed comparing the signals of that sample with data from GPRC5D and CD20 VLPs in an attempt to assign unfolding events

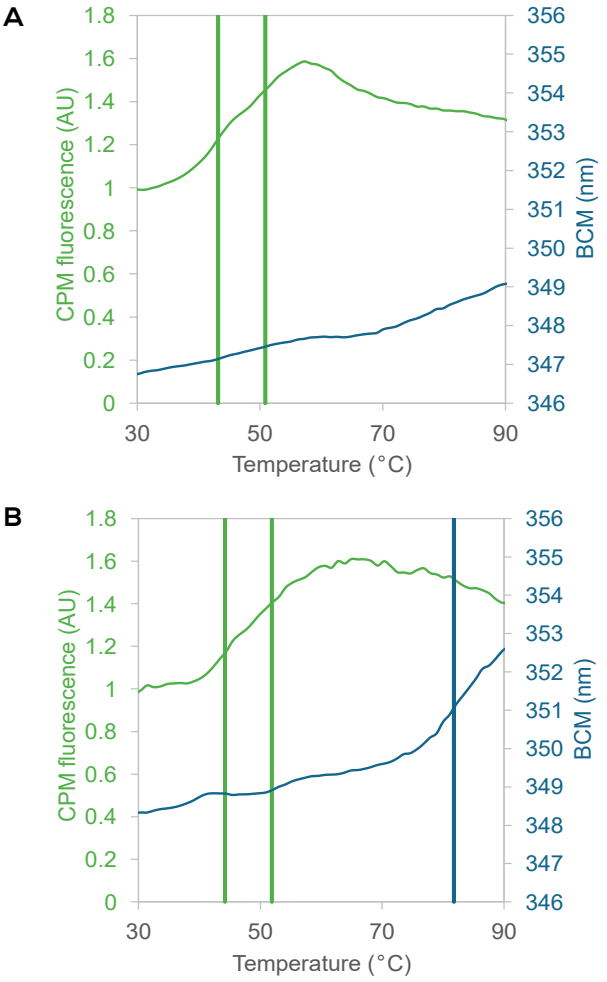

Figure 4: CD20 in its detergent formulation (**A**) had 2 T_ms based on the increase in CPM fluorescence (green); no T_m could be assigned based on its intrinsic fluorescence (blue). CD20 embedded in nanodiscs (**B**) had 2 CPM T_ms (green) and a single, much higher, intrinsic fluorescence T_m (blue). Representative fluorescence graphs of triplicate sample measurements are shown.

to each sample (Figure 5). The control VLP had 3 T_ms at 37.9, 48.5, and 59 °C. The GPRC5D VLP had 2 T_ms at 40.7, and 59.6 °C. The CD20 VLP had 2 T_ms at 46.4 and 56 °C. It's likely that the highest T_m from these samples represents the same viral protein unfolding event. Without additional experiments it is not possible to link specific unfolding events to specific proteins, but it is possible to make stability conclusions on the VLPs in total: GPRC5D VLP appears to be somewhat more stable than the VLP isotype control. CD20 also appears to make the same VLP less likely to unfold at lower temperatures.

Unfolding is a common way to rank protein thermal stability, but analyzing aggregation

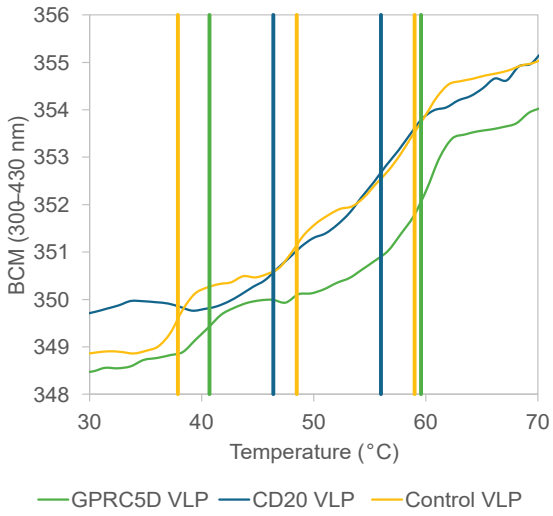

Figure 5: Intrinsic protein fluorescence can be used to characterize the stability of complex protein vehicles, including VLPs. GPRC5D (green), CD20 (blue), and control VLPs (yellow) had multiple T_ms based on the changes in their intrinsic fluorescence which are likely the result of several unfolding events of viral proteins and the protein cargos. Representative fluorescence graphs of triplicate measurements are shown.

presents another option. Just like it has multiple ways of detecting unfolding, Uncle has 2 orthogonal and complementary ways of detecting aggregation: DLS and SLS. DLS is useful for figuring out if the average size or size distribution of particles in a protein sample increased after heating, a common indicator of aggregation. Unlike in most proteins, the hydrodynamic diameter of GPRC5D in detergent stayed at approximately 100 nm after heat treatment (Figure 6A). Since the concentration of DDM in this sample was above the critical micelle concentration, these 100 nm particles likely represent GPRC5D in detergent micelles, not the protein alone. GPRC5D and CD20 VLPs decreased in size after heating. GPRC5D VLPs went from 236 to 186 nm and CD20 VLPs went from 158 to 136 nm. Heating is a common method to inactivate viruses and, based on the DLS and intrinsic fluorescence data, it's likely that the VLP underwent major structural changes in the heat treatment which may have also affected the membrane protein cargo.

SLS is an extremely sensitive way to detect aggregation during a thermal ramp and Uncle measures it simultaneously with fluorescence.

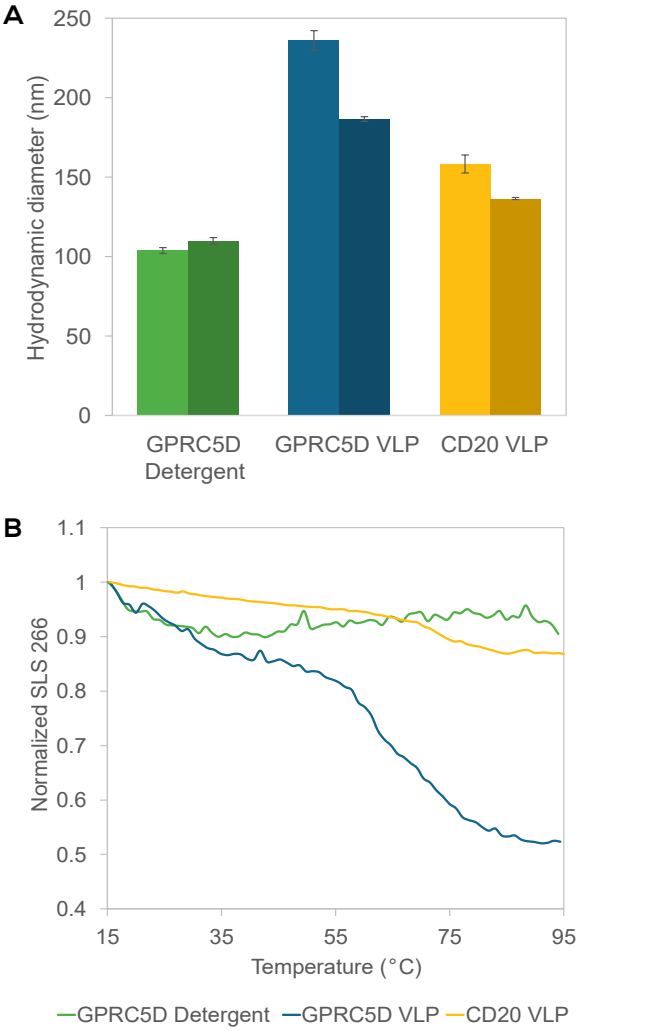

Figure 6: (**A**) GPRC5D in detergent had comparable hydrodynamic diameters before (light green) and after (dark green) heating. GPRC5D (blue) and CD20 (yellow) in VLPs were smaller after heating (light vs. dark). The SLS intensity (**B**) of GPRC5D in detergent remained stable during the thermal ramp while SLS of GPRC5D and CD20 VLPs decreased. The average of the hydrodynamic diameters of triplicates is shown with error bars equal to 1 standard deviation. Representative SLS curves from the triplicates are shown as normalized values to the initial SLS intensity.

When particles in a solution increase in mass, for example due to protein aggregation, the SLS intensity increases and when they shrink or disassemble, the SLS intensity decreases. The SLS intensity of the GPRC5D in detergent remained unchanged in a thermal ramp. In contrast, CD20 VLP SLS decreased with a significant drop starting at ~69 °C. GPRC5D VLP had a drop in SLS intensity at ~58 °C (Figure 6B). These SLS intensity drops indicate alterations in

the overall VLP structure, rather than changes to specific proteins. Combining Uncle's various thermal stability tools provides a much deeper understanding of the biophysical properties of complex vectors like VLPs than relying on a single technique.

Conclusion

Overexpressed membrane proteins are unstable and solubilization by detergents can exacerbate this problem. Stabilizing them under those conditions would simplify and accelerate the discovery and development of life-saving drugs, but traditional stability tools consume large amounts of sample and are difficult to adapt to high-throughput processes. Uncle tracks multiple stability parameters (fluorescence, SLS, and DLS), enabling it to deal with complex vehicles for membrane proteins including detergents, nanodiscs, or VLPs. Full-spectrum fluorescence gives you the flexibility to explore and find the optimal methods for examining your proteins, even if it involves using dyes like CPM. Uncle's low volume requirements make it so you can conduct tests more efficiently than ever before. SLS and DLS are orthogonal methods of detecting aggregation or size changes in general, and by combining size and unfolding information in Uncle it is possible to understand all the biophysical impacts heat can have on your membrane protein. With data like that you can tell not only what has happened to the protein but also draw conclusions as to why and develop strategies on what to do about it.

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