OPTIMIZATION OF MEMBRANE PROTEINS FOR CRYO-EM

Screening of copolymer nanodiscs with multi-parameter stability characterization on the Prometheus Panta

AUTHORS

Philipp T. Hanisch[1], Michael Ries[1], Greg Dodge[2], Léa Valla[3], Ping Zhang[3]

[1] Cube Biotech GmbH, Monheim-am-Rhein, Germany

[2] Massachussets institute of Technology (MIT), Imperiali Group, Boston, United States

[3] NanoTemper Technologies GmbH, Munich, Germany

ABSTRACT

Cryogenic Electron Microscopy (cryo-EM) has enabled atomic-level structure determination of many complex proteins that were not previously achievable with X-ray crystallography or nuclear magnetic resonance (NMR). Membrane proteins, while being critical targets for drug development, present additional challenges for structural analysis; their amphipathic nature, complex architecture, as well as tedious expression and purification continue to strain researchers working with these proteins. With the use of innovative solubilization approaches, such as synthetic copolymers and the NativeMP™ Platform, companies like Cube Biotech can more efficiently create soluble membrane proteins optimized for cryo-EM workflows to support structural biologists. In this work, we describe how the application of dynamic light scattering (DLS) and nano Differential Scanning Fluorimetry (nanoDSF) provided by the Prometheus Panta helps membrane protein structural biologists rapidly find the best copolymers for their synthetic nanodiscs to ensure the highest atomic resolution for cryo-EM structures.

KEYWORDS

Membrane proteins, cryo-EM, synthetic nanodiscs, copolymer, DLS, nanoDSF, Contract Research Organisation (CRO), NativeMP™

Introduction

Cryo-EM structural determination has vastly expanded the number and complexity of atomic-resolution structures available, facilitating a better understanding of mechanisms including cellular regulation that guide therapeutic development. Previously difficult-to-crystallize targets such as membrane proteins have become more feasible for structural analysis, despite their amphipathic nature, large size, and conformational flexibility. However, high-resolution cryo-EM studies depend on preparing grids with pure, monodisperse, well-folded proteins, which is still a significant hurdle to overcome. This challenge is especially pronounced for membrane proteins, as classical methods for solubilization and purification are laborious, and contain many potentially destabilizing steps[(1)].

Cube Biotech capitalizes on their expertise as a protein expression and purification service provider to optimize a wide variety of membrane protein classes. The company has found that their 100 percent detergent free NativeMP™ Platform, featuring a variety of synthetic copolymers derived from 5 different chemical backbones (SMA, DIBMA, AASTY, Ultrasolute™ Amphipol and Cubipol), are excellent reagents for solubilizing and stabilizing membrane proteins of all classes for cryo-EM based structure determination. The purified copolymer nanodiscs are composed of membrane proteins embedded within native cell membrane phospholipids. These complexes are held together by a synthetic copolymer ring, which offers improved stability when solubilizing membrane proteins for cryo-EM investigation.

Since Cube Biotech receives a large number and variety of membrane proteins for cryo-EM optimization, it is important to have high-throughput methods for optimizing which synthetic copolymers are best suited to the target proteins. They have found that sizing data provided by dynamic light scattering (DLS) measurements, along with conformational stability data from nano differential scanning fluorimetry (nanoDSF) obtained on the Prometheus Panta are incisive quality-control parameters for the copolymer screen. High-throughput optimization of this screening process also benefits academic researchers, by significantly reducing the amount of time and required material to identify optimal copolymers.

The Prometheus Panta is an instrument that utilizes label-free methods to simultaneously monitor the conformational and colloidal stability characteristics of proteins along a thermal denaturation gradient. This instrument uses very little material, requiring only 10 µL per sample (≥100µg/ml concentrated).

This small volume especially allows screening of different copolymers alongside many different membrane proteins using miniaturized expression and purification protocols.

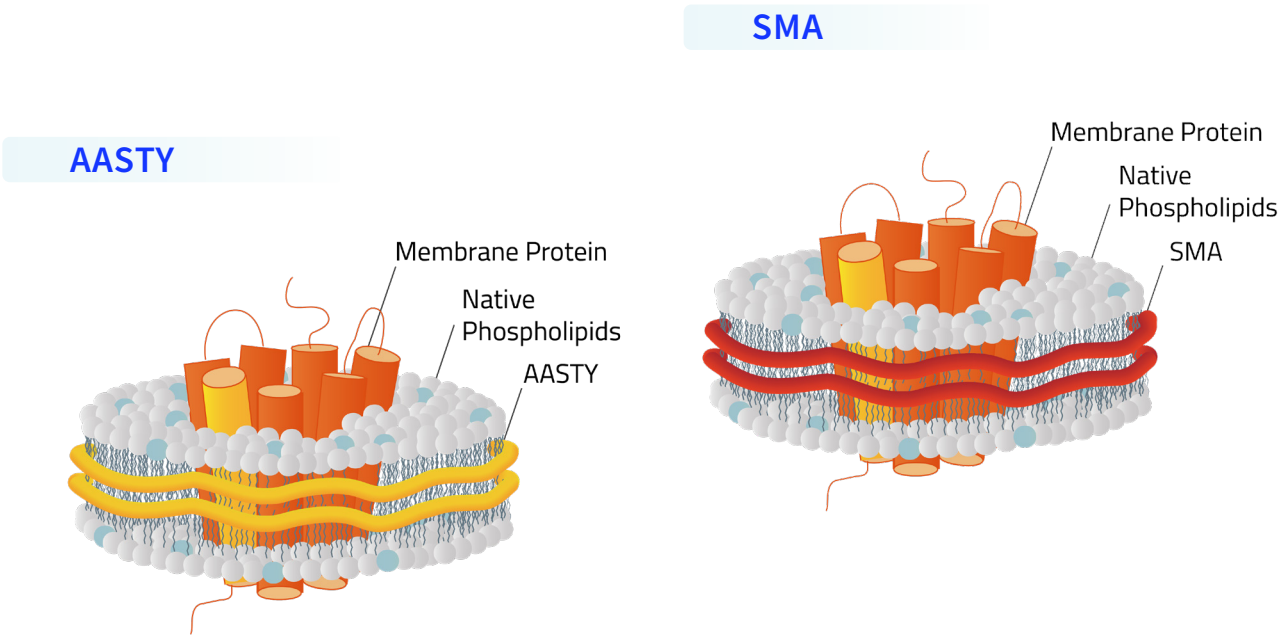
Isothermal DLS analysis provides information on the size (hydrodynamic radius, r_H) and the polydispersity index (PDI) of the membrane proteins stabilized in the nanodiscs, allowing the triage of copolymer nanodiscs with aberrant sizes. Next, nanoDSF, backreflection, and DLS data was collected along a temperature ramp on the same 10 µL samples to review the thermal stability of the candidates. Parameters such as melting temperature of the protein (T_m), the onsets of aggregation (T_{onset}Turbidity) and sizing increase ($T_{onset}r_H$) confirm the folding of the protein as well as the thermal stability of the nanodiscs.

The copolymers of the NativeMP™ Platform are commercially available and have been used for membrane protein studies all over the world. In particular, the group of Prof. Barbara Imperiali and postdoctoral researcher Dr. Greg Dodge at MIT had great success utilizing this platform. As illustrated in this application note, the combination of the NativeMP™ platform with the Prometheus Panta provided an optimal workflow for the prokaryotic membrane protein targets from the Imperiali group. The experiments shown in this application note have been performed by the Imperiali lab at MIT. More examples can be viewed in the webinar of Greg Dodge on the Cube Biotech Website.

Cube Biotech and the Imperiali Group validated that these parameters together allow rapid screening for optimal copolymers to solubilize a variety of membrane protein types, which was corroborated by information from SEC and SDS-PAGE. We anticipate that this workflow will be of general utility to the greater membrane protein community, especially for those interested in applications such as structural biology.

PROTEIN 1: WBAP COPOLYMER SCREEN

WbaP is a prototypic member of the large monotopic phosphoglycosyl transferase superfamily from *Salmonella enterica*. This protein catalyzes the transfer of galactose-1-phosphate onto undecaprenyl phosphate (Und-P) during the initial stage of lipopolysaccharide O-antigen biosynthesis and is a potential target for antibiotic development [2][3]. This class of bacterial proteins contains several subdomains, including a transmembrane four-helix bundle, a soluble nucleotide sensing domain, and a membrane-bound catalytic domain containing a re-entrant membrane helix[4]. The first cryo-EM structure of WbaP required extensive optimization of copolymer-based solubilization, ultimately leading to the development of this protocol.



Class of copolymers included in the Cube Synthetic Nanodisc screening Kit MAXI

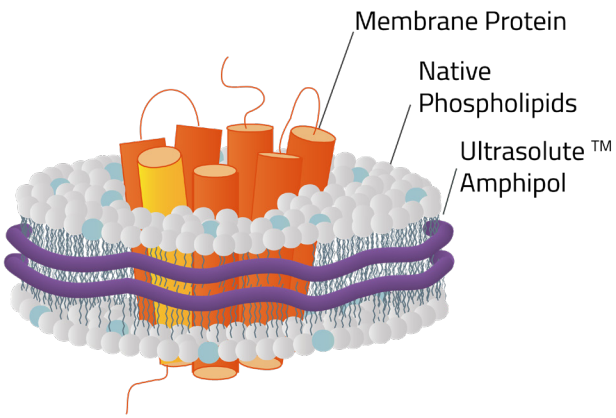
PROTEIN 2: PGLB BACKGROUND

PglB from *Neisseria Gonorrhoeae* contains a membrane-embedded phosphoglycosyl transferase domain fused to a soluble acetyltransferase (AT) domain and is involved in the production of extracellular polysaccharides. This bifunctional enzyme acetylates a UDP-sugar substrate, and then transfers the resulting acetylated phosphosugar onto Und-P. Structural studies on related ATs support that this domain mediates trimerization of the dual-function membrane protein. While there are structures of homologs for both subdomains, the structure of the full-length protein is of considerable interest for understanding the coordination of two sequential steps.

PROTEIN 3: PGLC BACKGROUND

PglC from *Helicobacter Pullorum* is a small membrane protein with an unusual topology, as it contains a single re-entrant membrane helix that enters and exits the bilayer on the same face. Like the above proteins, Hp PglC functions in the early stages of glycoconjugate biosynthesis, transferring the phosphosugar moeity from UDP-diNAcBac onto Und-P. While the small size of this protein (~20 kDa) precludes cryo-EM structure determination on its own, addition of mass fiduciaries in the form of fAbs, legobodies, or pro-macrobodies may represent a path to visualize the structure of this small protein in a native-like bilayer.

ULTRASOLUTE™ AMPHIPOL



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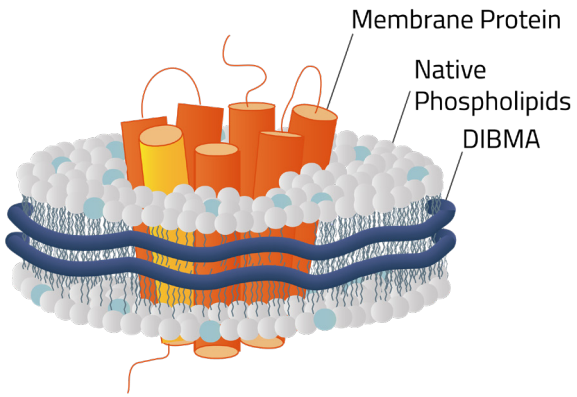


Class of copolymers included in the Cube Synthetic Nanodisc screening Kit MAXI

Results

PART 1: SIZING MEASUREMENTS WITH DLS

Isothermal DLS analysis using the Prometheus Panta was applied to all samples to study the size of the different copolymer nanodiscs stabilized membrane proteins. The hydrodynamic radius (r_H) of the major peak in the size distribution analysis reveals the size of the most populated particles in the sample. The typical desired radius of these nanodiscs is below 20 nm. Consequently, copolymers with an r_H value exceeding 20 nm (indicated in grey in Table 1) are initially excluded from further consideration.

As an example, here we demonstrate the overlayed size distribution plots of WbaP nanodiscs in Figure 1. Most of the samples exhibited a primary population of particle sizes with a peak around 10 nm (Figure 1A and Table 1). However, a few copolymer candidates demonstrated larger particle sizes of approximately 20-30 nm (Figure 1B and Table 1), which are unexpected for nanodiscs stabilizing a complex of the estimated size and therefore deselected from further study. In particular, sample 3-6, which consisted of particles with an r_H of approximately 30 nm, did not exhibit any bands of protein in the SDS-PAGE result (Figure 2). Additionally, samples 7, 8 and 14 display a band at the molecular weight of the protein as observed in the SDS-PAGE (Figure 2). However, the DLS results of these three samples reveal the presence of a considerable number of larger particles, making them unsuitable for further structural assays (Figure 1B, SMALP BZ25, SMALP BZ30 and AASTY 11-50 (1) respectively).

These observations demonstrate that DLS agrees with the results of SDS-PAGE in a copolymer screening workflow. Furthermore, it also exhibits the greater capabilities in the triage of additional candidates that may be overlooked by SDS-PAGE.

In conclusion, isothermal DLS can be used as a key parameter to select optimal candidate copolymers for downstream applications. Despite each copolymer candidate displaying distinctive characteristics in terms of solubilization efficiency and the r_H of the resulting nanoparticles, the Prometheus Panta system's low sample volume (10 µL) and short measurement time (50 seconds per sample), allows for rapid screening of dozens of copolymers at once. Optimal copolymers will produce nanoparticles with a uniform size distribution and low polydispersity. Protein-embedded SMALPS generally have a radius ~10nm, though there may be exceptions to this based on their cargo.

Table 1 Hydrodynamic radius of the major peak in DLS analysis of WbaP, PglB and Hp PglC nanodiscs

Index	Copolymer	r_H(nm)		
		WbaP	PglB	Hp PglC
1	SMA25 (SMA300)	7.46 ± 0.48	9.68 ± 0.45	9.59 ± 0.32
2	SMA30 (SMA200)	9.16 ± 0.72	8.71 ± 0.34	6.19 ± 1.54
3	SMA40 (SMA140)	32.18 ± 3.09	24.19 ± 2.83	22.18 ± 1.08
4	DIBMA 10	29.39 ± 2.39	29.10 ± 7.57	22.42 ± 2.5
5	Sulfo-DIBMA	30.07 ± 1.41	24.33 ± 2.47	23.29 ± 1.11
6	Sulfo-SMA	36.99 ± 2.34	27.23 ± 1.35	25.22 ± 1.8
7	SMALP BZ25	28.68 ± 10.76	8.48 ± 2.07	8.26 ± 1.53
8	SMALP BZ30	20.19 ± 4.59	9.91 ± 0.64	7.90 ± 2.52
9	SMALP BZ35	12.73 ± 1.19	9.73 ± 1.26	13.22 ± 4.32
10	SMALP BZ40	10.95 ± 0.49	10.21 ± 0.48	8.39 ± 0.79
11	AASTY 6-45	11.4 ± 0.47	10.84 ± 0.18	8.6 ± 0.43
12	AASTY 11-45	13.27 ± 4.39	8.44 ± 0.5	7.86 ± 1.72
13	AASTY 6-50	10.54 ± 2.08	8.92 ± 0.41	7.49 ± 1.17
14	AASTY 11-50 (1)	24.36 ± 7.89	8.47 ± 1.57	13.28 ± 4.18
15	AASTY 6-55	11.69 ± 2.51	8.68 ± 0.46	7.80 ± 1.73
16	AASTY 11-50 (2)	12.83 ± 3.20	8.35 ± 0.36	6.93 ± 1.33

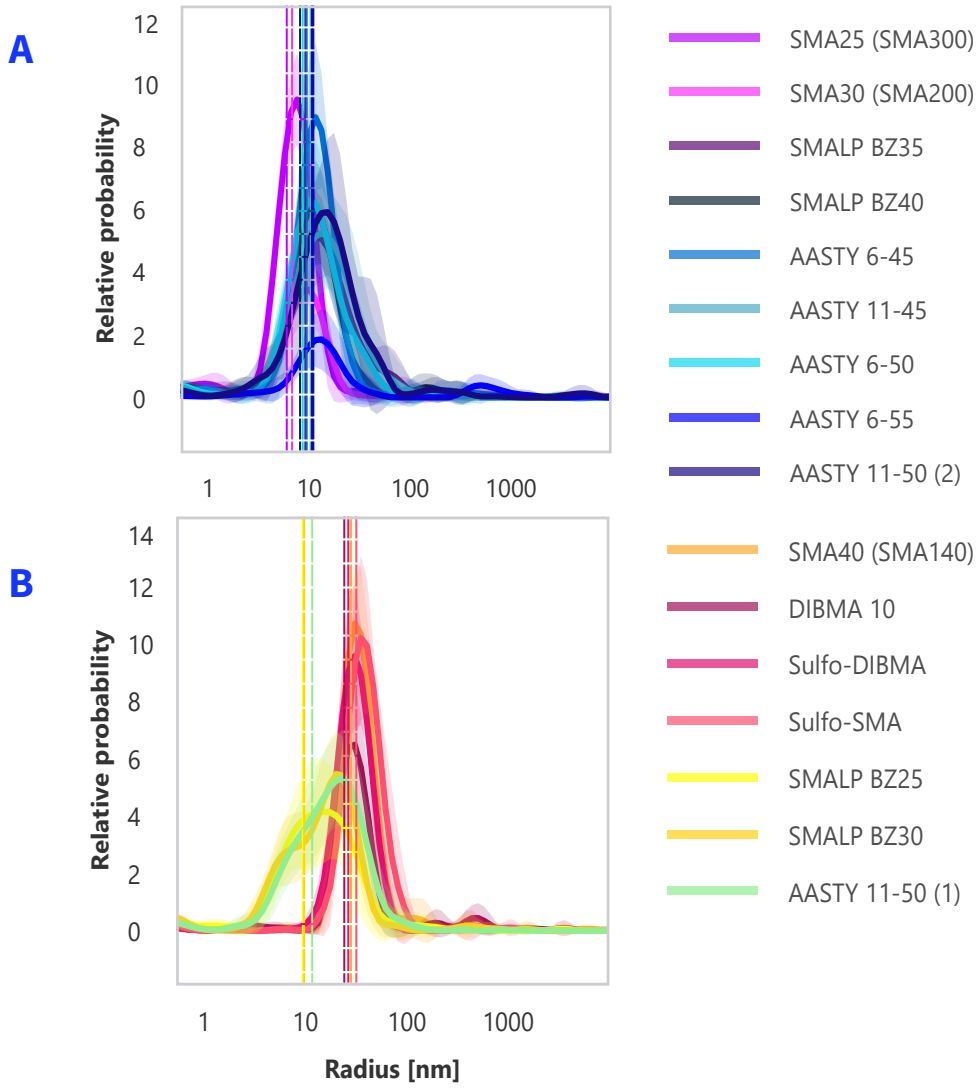
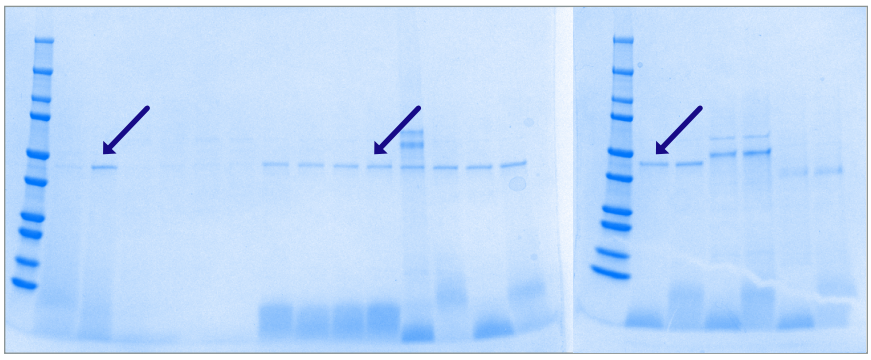


Figure 1: Overlayed DLS size distribution plots and cumulant radius (dash lines) for WbaP solubilized with 16 different copolymers.



SMA30	SMALP BZ40	AASTY 6-55
6.84 nm	8.1 nm	10.96 nm

Figure 2: SDS-PAGE gel of WbaP solubilized in each polymer. Each lane was loaded with 8 µL of eluate from strep resin. Lane 2 (SMA30), Lane 10 (SMALP BZ40), and Lane 15 (AASTY 6-55) are highlighted with arrows.

PART 2: THERMAL UNFOLDING ANALYSIS

Following the isothermal DLS measurements, a thermal ramp is applied to the same 10 µL of sample loaded in the capillary. nanoDSF uses the intrinsic fluorescence of the protein to gain insight into its conformational stability, while the simultaneous turbidity and DLS measurements evaluate the overall colloidal stability of the membrane protein-nanodisc complexes.

The first key parameter that was evaluated is the temperature of the onset of cumulant radius (T_{onset} r_H).

This is the temperature at which a size change for the population of nanodiscs is observed, indicating that the particles start losing structural integrity. Accordingly, this parameter was selected as the criterion for evaluating the thermal stability of the copolymer nanodiscs in their entirety. The respective values are presented in Table 2, with the samples with the three highest values highlighted in red for each membrane protein.

It is not sufficient to consider solely the thermal stability of the nanodisc structure. The copolymer must also be selected based on the thermal stability of the protein embedded in the nanodiscs.

NanoDSF measures the intrinsic fluorescence intensities of proteins at two wavelengths – 350 nm and 330 nm – with high precision to gain insight into the unfolding of proteins. When a protein unfolds in solution, a change in the environment of the tryptophan and tyrosine residues will result in a corresponding alteration in the fluorescent emission spectrum. This is represented by the fluorescence ratio (350 nm/330 nm) changes along a thermal ramp.

For a membrane protein stabilized by a synthetic copolymer nanodisc, the nanoDSF data does not necessarily exhibit a distinct unfolding transition in the fluorescence ratio, in contrast to the membrane protein solubilized in solution by detergent. This could be explained by two reasons. First, the structure of the nanodisc is often more thermally stable than the protein, which allows for protein unfolding to occur with minimal conformational changes while embedded in the nanodisc. Second, the high lipid content of the nanodisc limits the changes in hydrophobicity upon unfolding, thereby limiting the changes observed in the emission spectrum. Consequently, the unfolding can be observed in some cases by measuring the fluorescence intensity at 350 nm (as demonstrated by WbaP in Table 2 and Figure 3).

The inflection points of unfolding (equivalent to the melting temperature, T_m) for WbaP are listed in Table 2. Samples 1, 2, 9, 10 and 16, did not exhibit a distinct inflection point which can be attributed to the aforementioned reasons. However, for samples 11, 12, 13 and 15, a clear unfolding transition was observed by monitoring the fluorescence intensity changes at 350 nm, with the three highest inflection points highlighted in red in Table 2. In these samples, WbaP unfolds at around 55°C. However, the nanodisc disassembles at a temperature above 65°C, as evidenced by the r_H during the thermal gradient. By taking SDS-PAGE, isothermal DLS, and nanoDSF into account, AASTY 6-55 was identified as the optimal copolymer to solubilize and stabilize WbaP. The initial set of copolymers used for WbaP was significantly smaller than that used here and did not include any of the AASTY copolymers [2]. The results of this expanded copolymer panel demonstrate the utility of revisiting targets for screening with newly developed copolymers for high-value targets.

Unlike WbaP, which has minimal soluble domains, PglB has a large soluble acetyltransferase domain. A thermal ramp was also applied to PglB samples. The nanoDSF data for this group of samples yielded inconclusive results when analyzed using fluorescence intensity at a single wavelength or the ratio 350 nm/330 nm. However, the turbidity signal provides an effective and reliable method for examining the thermal stability of the protein. An increase in turbidity indicates the formation of large aggregates (>12.5 nm and >100 µg/mL). In this case, the unfolding of the soluble domain triggered the aggregation of the entire nanodisc, making it a suitable indicator for assessing the thermal stability of the protein.

This is illustrated by samples 12 and 13, presented in Figure 4. The turbidity signal increases in Sample 13 at 60.6°C, which contrasts with the lack of such an increase in Sample 12 throughout the thermal ramp. In Table 2, the three highest temperatures of onset of aggregation are indicated in red which demonstrates that no large aggregates were formed below 90°C. It can thus be deduced that the protein exhibits the highest thermal stability in these three nanodiscs. Together with the data obtained from the T_{onset} r_H, it becomes evident that the copolymer AASTY 11-45 exhibits a notable advantage in the overall stability when applied to PglB.

Finally, the nanoDSF data for the sample group PglC showed clear transitions in the fluorescence ratio 350 nm/350nm window along the thermal ramp. Therefore, the IPratio was identified as the second parameter in addition to the T_{onset} r_H for copolymer screening. A review of the data presented in Table 2 indicates that SMA30 (SMA200) is the most suitable copolymer for this protein. In comparison, the copolymer AASTY 11-45 nanodiscs demonstrated comparable thermal stability of the entire nanodisc, although the unfolding of the protein exhibited less stability than that observed with SMA30 (SMA200), with a difference of 9°C.

Looking at further copolymers screened with Hp PglC, additional information provided by the combination of simultaneous nanoDSF, DLS and backreflection measurements can be extracted and illustrated by a comparison of samples 9, 11, and 15 (SMA BZ35, AASTY 6-45, and AASTY 6-55). If all three copolymers show a similar T_{onset} r_H, the IP ratio for sample 9 is 8°C higher than for the other two copolymers. Moreover, as seen in Figure 5, turbidity measurements also show a different turbidity profile for sample 11 (AASTY 6-45), indicating an earlier onset for the formation of large aggregates.

Thus, the combined information allows for a ranking of those three copolymers with SMALP BZ35 being the most favorable and AASTY 6-45 the least favorable for stabilizing *Hp* PglC.

Table 2 Thermal stability of WbaP, PglB, and PglC nanodiscs

Index	Copolymer	WbaP		PglB		Hp PglC	
		T_{onset} r_H (°C)	IP_{350} nm (°C)	T_{onset} r_H (°C)	T_{onset} Turbidity (°C)	T_{onset} r_H (°C)	IP_{ratio} (°C)
1	SMA25 (SMA300)	**75.0**	N.A.	69.2	80.2	**69.8**	**68.0**
2	SMA30 (SMA200)	69.0	N.A.	77.2	**>90**	**>75**	**59.5**
3	SMA40 (SMA140)						
4	DIBMA 10						
5	Sulfo-DIBMA						
6	Sulfo-SMA						
7	SMA BZ25			59.7	76.2	49.9	55.4
8	SMALP BZ30			61.3	81.8	51.5	55.7
9	SMALP BZ35	52.2	N.A.	65.5	81.8	51.1	56.8
10	SMALP BZ40	N.A.	N.A.	50.1	80.0	48.8	**57.2**
11	AASTY 6-45	65.7	**57.5**	46.0	50.2	51.1	48.2
12	AASTY 11-45	N.A.	51.8	**87.3**	**>95**	**69.6**	50.6
13	AASTY 6-50	68.5	**55.5**	56.0	60.6	47.3	48.3
14	AASTY 11-50 (1)			**80.4**	**>95**	62.3	50.2
15	AASTY 6-55	**74.7**	**53.9**	62.0	86.2	51.5	48.9
16	AASTY 11-50 (2)	**79.7**	N.A.	73.0	**>90**	59.6	47.0

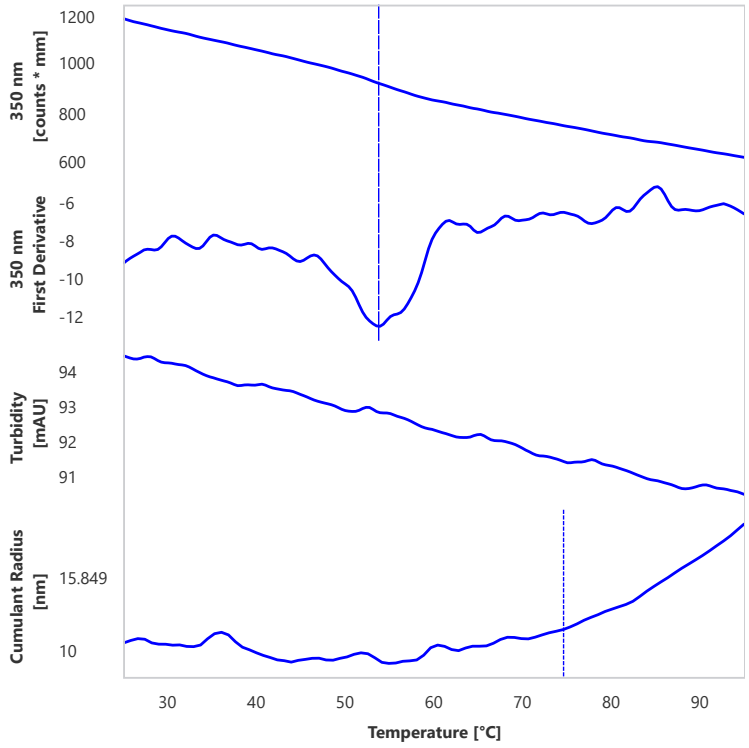


Figure 3: Thermal unfolding analysis of WbaP stabilized by AASTY 6-55 nanodisc.

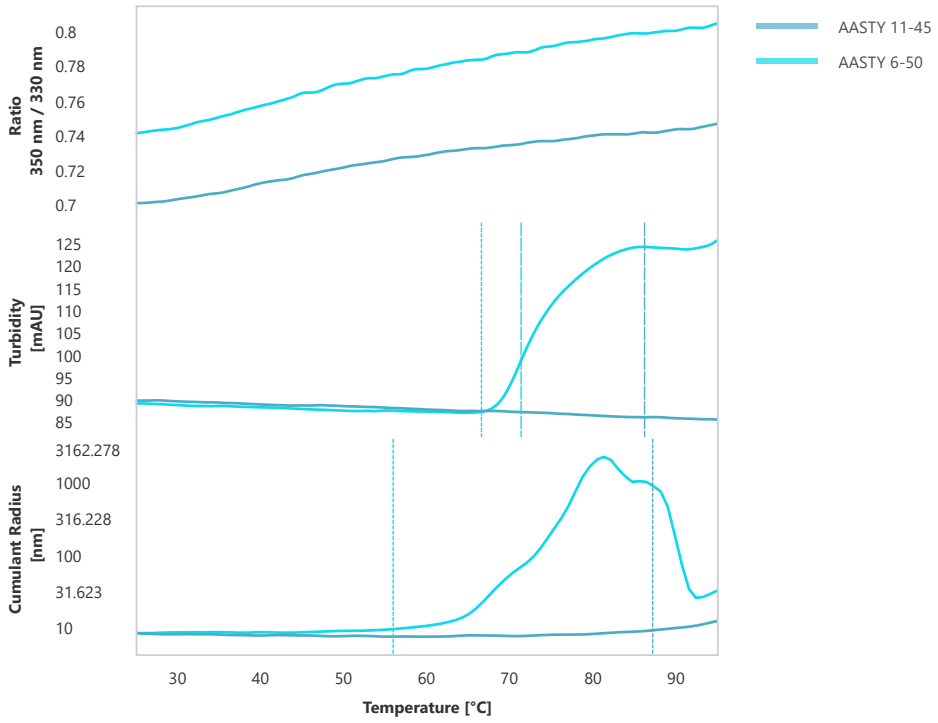


Figure 4: Thermal unfolding analysis of PglB stabilized by AASTY 11-45 (PglB 12) and AASTY 6-50 (PglB 13) nanodiscs across a thermal ramp.

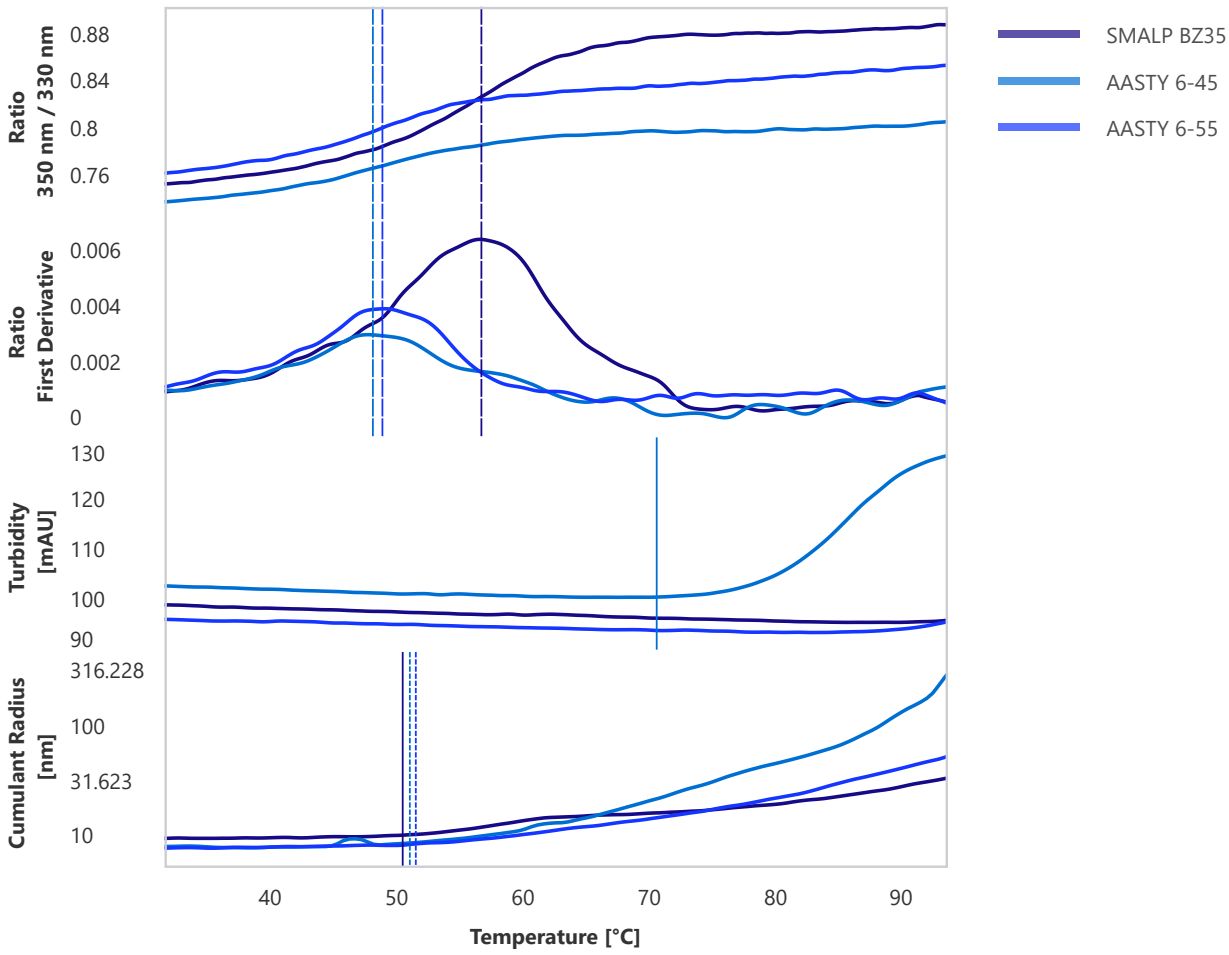


Figure 5: Thermal unfolding analysis of Hp PglC stabilized by SMALP BZ35, AASTY 6-45, and AASTY 6-55 nanodiscs.

METHODS

Protein expression and purification

All proteins were recombinantly expressed in E. coli and purified using an optimized protocol described previously[2]. Strep-Tactin®XT Resin was used to purify the membrane protein, which was solubilized in copolymer nanodiscs from the Cube Synthetic Nanodisc Screening Kit MAXI.

Prometheus Panta experiments

To identify the most effective copolymer for stabilizing and isolating membrane proteins in their active states, a series of miniaturized expression and purification experiments was conducted using 16 copolymers of the NativeMP™ platform on the three protein classes represented by WbaP, PglB and PglC (Table 1). After each miniaturized purification, 10 µL of sample was directly loaded into Prometheus Standard Capillaries, and the isothermal (25 °C) DLS measurements were conducted using the Prometheus Panta. Subsequently, a thermal ramp (1.5 °C/min, 25-95 °C) was applied to each 10 µL sample, with the nanoDSF, DLS, and turbidity data being collected simultaneously.

Summary

The data shown in the three examples above highlights the importance of screening a wide range of copolymers for finding the optimal nanodisc to solubilize and stabilize membrane proteins. Such samples are suitable for downstream applications such as cryo-EM, enzyme assays, or protein binding studies.

The specific characteristics of each copolymer class may confer advantages to a particular membrane protein, however there is no definitive rule that can be universally applied to identify the optimal candidate.

Due to its high throughput and low sample consumption, the Prometheus Panta can be used in the miniaturized expression and purification protocol co-proposed by Cube Biotech and the Imperiali lab to quickly screen a variety of copolymers that will confer optimal properties for both thermal and colloidal stability of membrane proteins embedded in native nanodiscs, allowing for further successful cryo-EM structure determination[2].

In particular, isothermal DLS measurements utilizing as little as 10 µL of sample can be employed to initially efficiently triage potential copolymers in accordance with the sizing information, without the risk of false-positive results due to the heterogeneity of the sample or false-negative results due to proteolysis which occur in the SDS-PAGE.

Furthermore, using the same 10 µL of sample, the combined measurements of nanoDSF, DLS, and backreflection across a thermal ramp from the Prometheus Panta give additional insight into the conformational stability of the protein and the overall colloidal stability of the nanodiscs, thus narrowing down the selection.

We have demonstrated that the NativeMP™ Platform offered by Cube Biotech, combined with the time efficient, miniaturized expression and purification of the membrane proteins, and the use of the Prometheus Panta instrument for screening and characterization of the different native nanodiscs represents an efficient workflow to find optimal conditions for cryo-EM experiments with membrane proteins, minimizing the amount of time and sample needed.

References

1. Kampjut et al., “Cryo-EM grid optimization for membrane proteins” iScience 24, 102139 March 19, 2021, https://doi.org/10.1016/j.isci.2021.102139

2. Greg J. Dodge, Hannah M. Bernstein, Barbara Imperiali. “A generalizable protocol for expression and purification of membrane-bound bacterial phosphoglycosyl transferases in liponanoparticles.” Protein Expression and Purification, Volume 207, 2023, 106273. https://doi.org/10.1016/j.pep.2023.106273.

3. Greg J Dodge, Alyssa J Anderson, Yi He, Weijing Liu, Rosa Viner, Barbara Imperiali (2024) “Mapping the architecture of the initiating phosphoglycosyl transferase from S. enterica O-antigen biosynthesis in a liponanoparticle.” eLife 12:RP91125. https://doi.org/10.7554/eLife.91125.2

4. Ray, L.C., Das, D., Entova, S. et al. “Membrane association of monotopic phosphoglycosyl transferase underpins function.” Nat Chem Biol 14, 538–541 (2018). https://doi.org/10.1038/s41589-018-0054-z