APPLICATION NOTE

ACHIEVE HIGHLY SENSITIVE AND PRECISE BINDING AFFINITY MEASUREMENTS WITHOUT DISRUPTING PROTEIN CONFORMATION

Redefine Membrane Protein Research Utilizing NativeMP™ Technology and the Monolith X

KEYWORDS

Membrane proteins
Synthetic nanodiscs
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ABSTRACT

Membrane proteins are critical in drug discovery due to their central role in cellular communication and signaling, making them key therapeutic targets for next generation medicines. However, their unique properties pose significant challenges for biophysical characterization, particularly in maintaining stability and native conformation outside of biological membranes. The stringent buffer conditions required to maintain the integrity of membrane proteins when investigating their structure and function are limiting, highlighting the need for innovative technologies that don't rely on traditional detergent-based approaches to facilitate novel drug discovery and drug interaction analysis with membrane proteins.

This application note underscores the combined strength of using the latest technologies from Cube Biotech and NanoTemper Technologies to supersede these challenges. NativeMP™ Technology, developed by Cube Biotech, utilizes detergent-free synthetic copolymers to stabilize membrane proteins in their native environment, while the Monolith X instrument from NanoTemper Technologies employs Spectral Shift and TRIC technologies to directly measure biomolecular interactions in solution; coupling these technologies empowers researchers to obtain highly sensitive and precise binding affinities while preserving the protein's native conformation during measurements.

Introduction

Membrane proteins are pivotal to cellular communication and signal transduction, making them critical targets in drug discovery. Their involvement in numerous physiological and pathological processes underscores their functional importance. However, the biophysical characterization of membrane proteins poses unique challenges due to low expression levels, inherent instability outside biological membranes, and the necessity of preserving their native lipid environment for functional studies. Traditional detergent-based solubilization strategies often disrupt membrane protein structure and function, complicating the interpretation of experimental results.

Cube Biotech's NativeMP™ Technology offers advanced synthetic copolymers, including Ultrasolute™ Amphipols (CyclAPols), AASTYs, Cubipols, SMAs, and DIBMAs, to stabilize membrane proteins in their native environment without the use of detergents. This preserves their structural integrity and associated lipid milieu, providing a robust foundation for reliable biophysical assays. Expanding upon previous studies that combined the strengths of NativeMP™ Technology with the capabilities of NanoTemper Technologies' Prometheus Panta instrument for stability assessments, this application note investigates the integration of NativeMP™ Technology with NanoTemper Technologies' Monolith X instrument for precise binding affinity characterization.

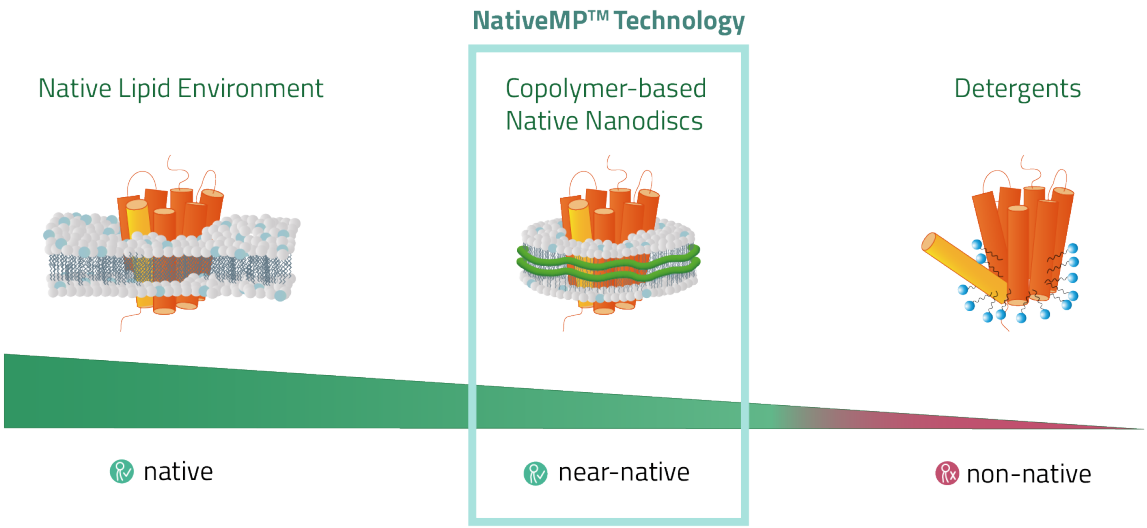

Figure 1: Class of copolymers included in the NativeMP™ Screening Kit.

Ensuring that membrane proteins retain their native conformation throughout biophysical analysis is paramount to study their structure-function interactions. The Monolith X instrument measures mass-independent biomolecular interactions in solution, with any buffer composition including excipients, and using minimal sample; these attributes are idyllic for a variety of membrane protein-related interactions, including protein-small molecule, protein-peptide, and protein-protein interactions that have proven difficult using other technologies.

In the following sections, this application note will underscore specific examples that illustrate the implementation of combining NativeMP™ Technology and the Monolith X instrument for membrane protein interaction studies, highlighting the effectiveness and versatility of this application for advancing biophysical characterizations and accelerating new therapeutic discoveries.

Figure 2: Monolith X with spectral Shift technology for binding affinity studies.

Membrane protein – Small molecule interaction: P2X4 and 5-BDBD

The P2X purinoceptor 4 (P2X4), a member of the P2X family of ligand-gated ion channels, plays a crucial role in mediating the cellular response to extracellular adenosine triphosphate (ATP) (1). Biologically, P2X4 receptors have a trimeric structure and are permeable to cations such as calcium (Ca^{2+}), sodium (Na^{+}), and potassium (K^{+}), which can initiate various intracellular signaling cascades (2,3). The receptor is widely expressed in immune cells, such as microglia, neurons, and endothelial cells, where it influences processes like neurotransmission, inflammation, and immune response (4). One key aspect of its function is in the central nervous system, where P2X4 is implicated in synaptic plasticity and neuropathic pain pathways (5), by promoting the release of pro-inflammatory cytokines and other mediators from microglia.

From a therapeutic perspective, the P2X4 receptor has garnered considerable interest as a drug target due to its involvement in pathological conditions, particularly neuropathic pain and inflammation (5). Its role in microglia activation, especially in the spinal cord during chronic pain states, has highlighted its relevance in developing novel pain therapeutics (6). Given its role in mediating neuropathic pain and inflammation, the discovery of P2X4 antagonists could lead to new therapies for chronic pain conditions that are resistant to current treatments. These molecules could selectively block the receptor's activity in microglia, thereby reducing the release of pro-inflammatory cytokines and attenuating pain signaling pathways.

Membrane protein – Peptide interaction: GLP1R and Exendin 9-39

The Glucagon-Like Peptide-1 Receptor (GLP1R) plays a crucial role in managing blood sugar and maintaining energy balance. It is part of the G-protein-coupled receptor (GPCR) family, which helps transmit signals across cell membranes. GLP1R is mainly found in pancreatic β-cells, where its activation triggers insulin release when blood sugar levels rise. It also lowers glucagon secretion, a hormone that increases blood sugar, and slows down stomach emptying, helping to control post-meal glucose levels (7). Beyond regulating glucose, GLP1R is also present in the brain, where it affects appetite, and in the cardiovascular system, offering potential heart benefits (8).

Exendin (9-39), a GLP1R antagonist, shows therapeutic potential in various conditions. It effectively raises blood glucose by inhibiting excessive insulin secretion in Congenital Hyperinsulinism (CHI), offering hope for patients unresponsive to standard therapies. Preclinical studies suggest it may influence neuroprotective mechanisms, impacting synaptic transmission and memory through glutamate uptake and astrocytic regulation. Additionally, Exendin (9-39) has been explored as a probe for imaging pancreatic beta-cells, aiding in understanding pancreatic function and beta-cell distribution in conditions like diabetes.

Membrane protein – Membrane protein interaction: NTCP and LHBsAg

Hepatitis B Virus (HBV) infection is a major global health issue, leading to severe liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) (17). The entry of HBV into hepatocytes is mediated by the sodium taurocholate co-transporting polypeptide (NTCP), which serves as a receptor for the virus. This interaction occurs specifically between NTCP and the large hepatitis B surface antigen (LHBsAg), facilitating viral attachment and internalization (18). NTCP is a transmembrane protein that primarily functions as a bile acid transporter in the liver. It contains several glycosylation sites that are crucial for its stability, trafficking, and role in HBV infection (19). The full-length structure of NTCP has not yet been resolved, but its functional domains involved in viral entry have been characterized through mutational studies, which indicate critical residues like tyrosine 146, and are necessary for HBV binding and internalization (20).

The large hepatitis B surface antigen (LHBsAg) is a glycoprotein on the viral envelope that mediates the initial attachment to hepatocytes through its pre-S1 domain. Although the full-length structure of LHBsAg is not completely solved, its interaction domains with NTCP have been identified (21).

Results

Following the screening approach outlined in the previous app note, the most suitable co-polymers were identified and used in the purification of GLP1R, P2X4, NTCP, and L-HBsAg respectively using the NativeMP™ Platform and Prometheus Panta. The quality and stability of the purified full length membrane proteins was validated through Size Exclusion Chromatography, SDS-PAGE, Western Blot as well as DLS and nanoDSF via the Prometheus Panta prior to the interaction analysis.

P2X4 – small molecule interaction

Therapeutic opportunities involving the P2X4 receptor are promising, particularly through the development of small molecule modulators. High-throughput screening of small molecule binders to P2X4 offers a powerful strategy to identify compounds that can either inhibit or enhance the receptor's function. The present case study examined the interaction between the P2X4 receptor and the small molecule antagonist 5-BDBD (5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one).

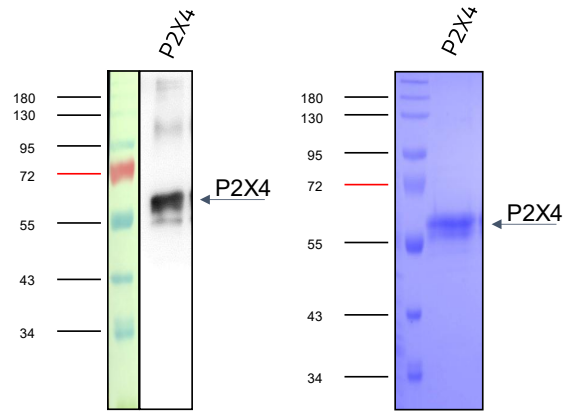

Figure 3: Western blot and SDS PAGE of P2X4 solubilized with Native MP™. P2X4 with a theoretical size of 44,6 kDa was detected at an apparent height of ~ 60 kDa (marked with arrows). Detection of proteins in SDS Page via specific primary Rho1D4 antibody and secondary HRP antibody.

As illustrated in Figure 4A, the copolymer nanodisc-stabilized P2X4 had a hydrodynamic radius (rH) of 10.81 ± 0.45 nm, which aligns with the anticipated size of the nanodisc. The polydispersity index (PDI) was demonstrated to be 0.24 ± 0.02, indicating that the purified sample was monodisperse and thus well suited for further biophysical analysis. In addition, DLS analysis of the same sample in a thermal ramp also shows that the nanodisc-stabilized P2X4 remained thermally stable up to approximately 55°C.

Following confirmation of the sample quality, the interaction analysis was conducted using the Monolith X as described in the method section. The Cy5 analogue-labeled P2X4 nanodisc was maintained at a constant concentration of 20 nM, while the concentration of 5-BDBD was varied between 2 nM and 45 µM (Figure 4B). The Kd was determined by analyzing the Spectral Shift signal - ratio of fluorescence intensities at 670 and 650 nm - yielding a value of 4.29 µM which aligns with the published data (24,25). It is also noteworthy that the signal-to-noise ratio (S/N) of the Kd fit in Figure 4B has a value of 14. The S/N value is a measure of the binding response amplitude relative to the noise level of the replicates. An S/N value greater than 12 indicates an excellent condition for detecting the binding.

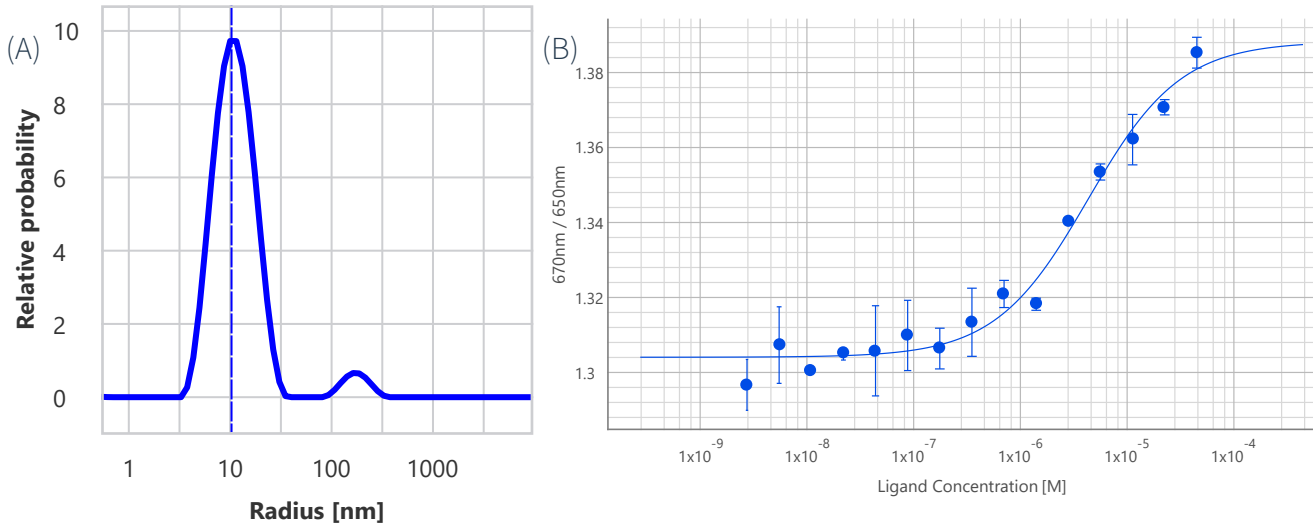

Figure 4: **(**A). DLS analysis of copolymer nanodisc-stabilized P2X4. The cumulant analysis reported the polydispersity index (PDI) of the sample to be 0.24 ± 0.02. The size distribution analysis reported the hydrodynamic radius (rH) of the P2X4 nanodisc to be 10.81 ± 0.45 nm. (B). Binding of antagonist 5-BDBD to nanodisc-stabilized P2X4. The fluorescently labeled P2X4 was used at a constant concentration of 20 nM with varying concentrations of the 5-BDBD in 20 mM HEPES pH 7.4, 150 mM NaCl, and 0.005% Tween 20. Ratios of the fluorescence intensities at 670 and 650 nm were used for interaction analysis yielding the Kd of 4.29 µM with S/N ratio of 14. Error bars represent the standard deviation of 2 independent measurements and with a confidence of 95%, Kd is within 2.87-6.42 µM.

In summary, the copolymer nanodisc-stabilized P2X4 exhibited excellent colloidal and thermal stability which is essential for interaction analysis. The Spectral Shift method was selected as the optimal approach for analyzing the binding of a small molecule (~355.19Da) to a large nanodisc (~150 kDa trimeric P2X4 + surrounding lipids and copolymer belt) due to its sensitivity and precision in detecting biomolecular interactions regardless of mass changes upon binding. This case study highlights the potential of utilizing the aforementioned system in a membrane protein ligand screening project.

GLP1R – Exendin (9-39) peptide interaction

Exendin (9-39) (Avexitide), a truncated form of the GLP1R agonist, exendin-4, is a specific GLP-1 receptor antagonist which is known to prevent hypoglycemia and maintains stability of blood glucose during a prolonged fast in individuals with KATPHI (congenital hyperinsulinism owing to inactivating mutations in the ATP-sensitive K+ channel) (23).

As illustrated in Figure 6A, the copolymer nanodisc-stabilized GLP1R had a hydrodynamic radius (rH) of 5.51 ± 0.12 nm, which aligns with the anticipated size of the GPCR-lipid-nanodisc complex. The polydispersity index (PDI) was determined to be 0.06 ± 0.02, indicating that the purified sample was highly monodisperse. In addition, DLS analysis of the same sample in a thermal ramp also shows that the nanodisc-stabilized GLP1R remained thermally stable up to approximately 58°C.

The Cy5 analogue-labeled GLP1R nanodisc was maintained at a constant concentration of 20 nM, while the concentration of Exendin (9-39) was varied between 0.02 nM and 0.4 µM (Figure 6B). The Kd was determined by analyzing the Spectral Shift signal yielding 4.51 nM, which aligns with the published data.

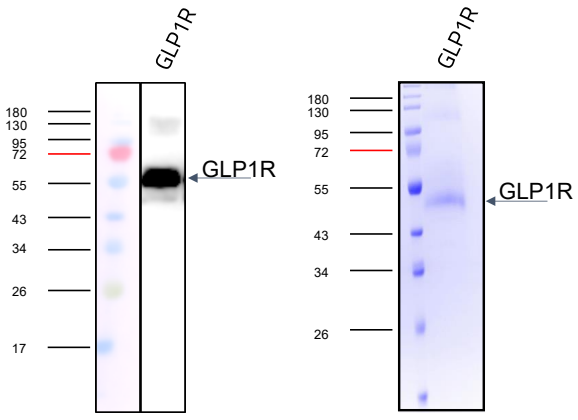

Figure 5: Western blot and SDS PAGE of GLP1R solubilized with Native MP™. GLP1R runs at a height of its theoretical molecular weight of 56.17 kDa (marked with arrows). Detection of proteins in SDS Page via specific primary Rho1D4 antibody and secondary HRP antibody.

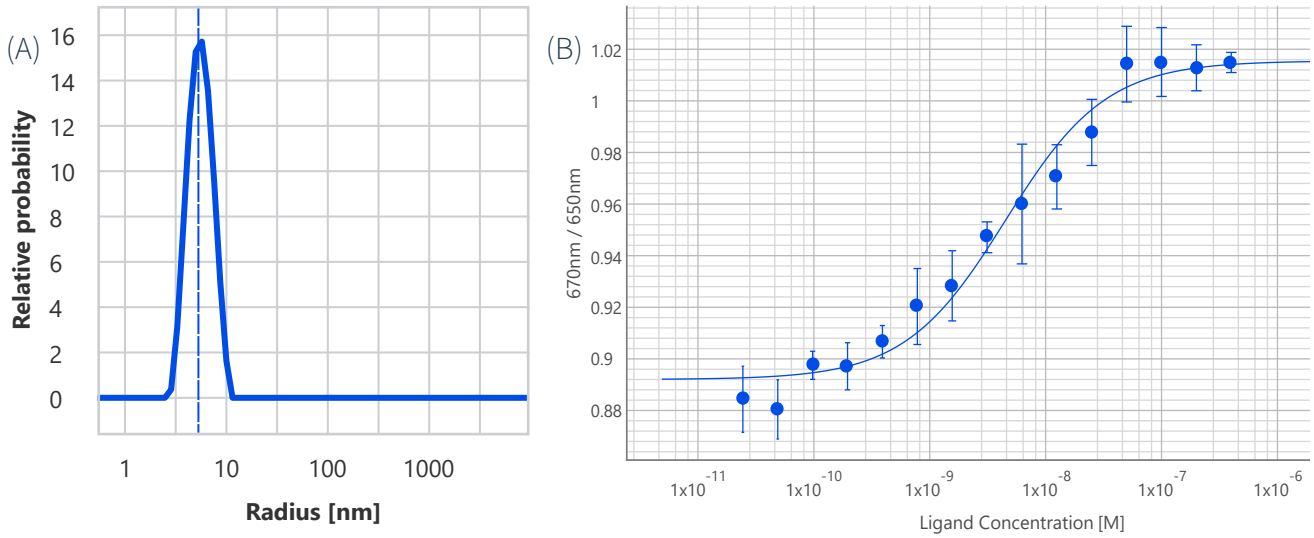

Figure 6: (A) DLS analysis of copolymer nanodisc-stabilized GLP1R. The cumulant analysis reported the polydispersity index (PDI) of the sample to be 0.06 ± 0.02. The size distribution analysis reported the hydrodynamic radius (rH) of the GLP1R nanodisc to be 5.51 ± 0.12 nm. (B). Binding of Exendin (9-39) to nanodisc-stabilized GLP1R. The fluorescently labeled GLP1R was used at a constant concentration of 20 nM with varying concentrations of the Exendin (9-39) in 20 mM HEPES pH 7, 150 mM NaCl, and 0.005% Tween 20. Ratios of the fluorescence intensities at 670 and 650 nm were used for interaction analysis yielding the Kd of 4.51 nM with S/N ratio of 10.1. Error bars represent the standard deviation of 3 independent measurements and with a confidence of 95%, Kd is within 1.79-5.91 nM.

The copolymer nanodisc-stabilized full-length GPCR, GLP1R, was found to be highly monodisperse and thermally stable. Using this high-quality protein sample, the Spectral Shift measurement was successfully employed to study the binding of a peptide to the membrane nanodiscs in the assay buffer without further optimization steps. The Monolith X is designed for user-friendly operation, offering a straightforward assay setup and intuitive software that provides detailed guidance to users. This ease of use makes interaction analysis accessible to interdisciplinary scientists and enables biophysical experts to be more efficient, especially when working with challenging protein targets.

NTCP and LHBsAg

Gaining more insights into the NTCP-LHBsAg interaction is critical for developing new therapeutic strategies, as it could lead to targeted treatments that prevent HBV entry into hepatocytes and reduce the viral load in infected individuals. Understanding this interaction in detail could also provide valuable information for combating HBV-associated conditions such as cirrhosis and liver cancer (22). This is the first study in which both proteins were purified as unaltered full-length versions to keep the approach as native as possible.

As illustrated in Figure 8A, the copolymer nanodisc-stabilized NTCP and LHBsAg had a hydrodynamic radius (rH) of 6.57 ± 0.22 nm and 5.80 ± 0.29 nm respectively. The polydispersity index (PDI) was demonstrated to be 0.20 ± 0.04 and 0.16 ± 0.04 for NTCP and LHBsAg. The sizing information confirmed the anticipated formation of the nanodiscs, and the PDI indicated that the purified samples were monodisperse for further biophysical analyses. Both samples remained thermal stable up to approximately 53 °C (NTCP) and 79°C (LHBsAg).

The Cy5 analogue-labeled NTCP nanodisc was maintained at a constant concentration of 20 nM, while the concentration of non-labeled L-HBsAg nanodisc was varied between 0.2 nM and 9.3 µM (Figure 3B cyan). A decrease of nanodiscs with L-HBsAg inside was complemented with empty nanodiscs to preserve its constant molarity while only L-HBsAg had changing concentrations. The Kd was determined by analyzing the TRIC signal using 670 nm at 2.5 s yielding a Kd of 967 nM. The black data shows the negative control using the same assay settings only with a dilution series of a non-specific membrane protein.

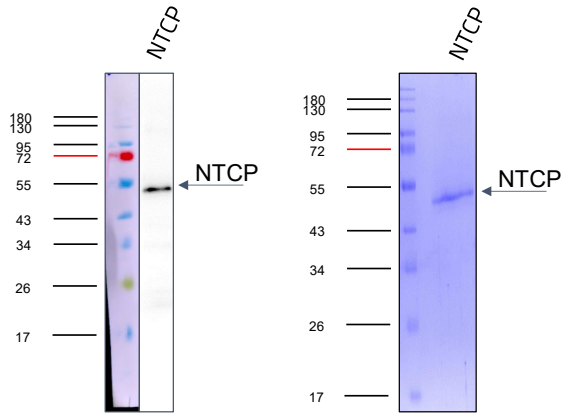

Figure 7: Western blot and SDS PAGE of NTCP solubilized with Native MP™. NTCP is a protein with a theoretical size of 39,3 kDa and was detected at an apparent height of ~ 48 kDa (marked with arrows). Detection of proteins in SDS Page via specific primary Rho1D4 antibody and secondary HRP antibody.

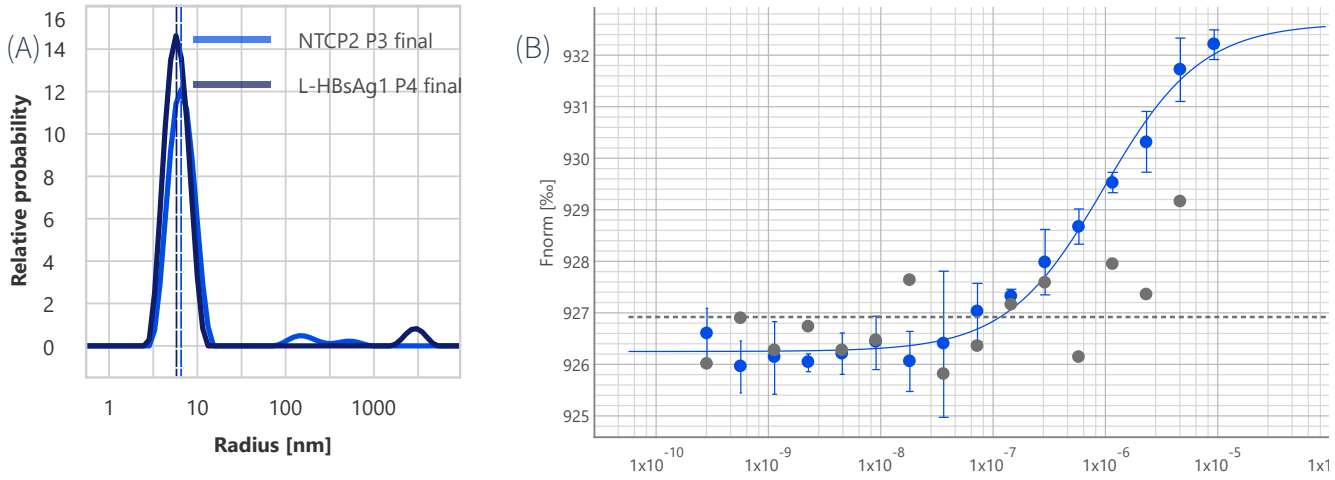

Figure 8: (A) DLS analysis of copolymer nanodisc-stabilized NTCP (cyan) and LHBsAg (purple). The cumulant analysis reported the polydispersity index (PDI) of the sample NTCP and LHBsAg to be 0.20 ± 0.04 and 0.16 ± 0.04 respectively. The size distribution analysis reported the hydrodynamic radius (rH) to be 6.57 ± 0.22 nm and 5.80 ± 0.29 nm for the NTCP and LHBsAg nanodiscs. (B). Binding of nanodisc-stabilized LHBsAg to nanodisc-stabilized NTCP (cyan). The fluorescently labeled NTCP was used at a constant concentration of 20 nM with varying concentrations of the LHBsAg in 20 mM HEPES pH 7.5 and 150 mM NaCl. Fluorescence intensity at 670 was used to get TRIC response (on time 2.5s) for interaction analysis yielding the Kd of 967 nM with S/N ratio of 12.78. Error bars represent the standard deviation of 3 independent measurements and with a confidence of 95%, Kd is within 669 - 1396 nM. The black data shows the negative control using the labeled NTCP mixed with a dilution series of a non-specific membrane protein.

The interaction studies between two full-length membrane proteins presents a significant challenge for researchers. The utilization of nanodiscs obviates the necessity to consider the detergents required for each membrane protein. In this case study, the interaction is observed exclusively through TRIC detection. TRIC and Spectral Shift are essentially two orthogonal biophysical methods based on two biophysical principles. When investigating an unknown interaction or working with an unconfirmed assay condition for the first time, combining two biophysical principles in a single measurement on a single instrument represents a great advantage, enhancing the success of interaction analysis.

Material and Methods

Protein expression and purification

All membrane proteins were either expressed in HEK293 or T.ni. cells and purified via a C-terminal Rho1D4 Tag utilizing HighSpec Rho1D4 MagBeads (Cube Biotech Order number: 33205). The detailed purification protocol ("Membrane Protein Solubilization Protocol with Copolymers") can be accessed via the Cube Biotech Website.

In short: Cells were thawed in buffer with protease inhibitor cocktail, lysed via sonication. Cell debris were separated via centrifugation, then the membrane fraction was purified via ultracentrifugation. Membrane pellets were solubilized via NativeMP™ Copolymer (best fit predetermined experimentally) and subsequently bound to Rho1D4 MagBeads for 3h. Purified target protein was eluted via HighSpec Rho1D4 Peptide (Order number: 16201), concentrated to 350 µl and run over Superose 6 10/300GL Size Exclusion Chromatography. Fractions were analysed via Prometheus Panta, SDS-PAGE and Western Blot. Fractions of interest were pooled and used for analysis.

Monolith X experiments

Covalent labeling of lysine residues of each membrane protein was performed using commercially available Cy5 analogues with a degree of labeling of approximately 0.5-1. For the Monolith X measurement, each ligand dilution (16 of 1:1 dilutions) was mixed with 40 nM labeled protein, which led to a final concentration of protein of 20 nM. After 30 min incubation, the samples were loaded into Monolith Capillaries (cat# MO-K022, NanoTemper Technologies). Spectral Shift measurements were performed at 25°C using the Monolith X instrument (NanoTemper Technologies). Instrument parameters were adjusted to auto-excitation power and medium IR laser power. Spectral Shift (Ratio 670/650 nm) or TRIC (ΔFnorm) of all ligand concentrations were calculated automatically within the MO.Control software (v2.6.3, NanoTemper Technologies GmbH). Data of independently pipetted measurements were analyzed using the MO.Control software (v2.6.3, NanoTemper Technologies GmbH).

Conclusion

Quantifying binding affinities is fundamental for understanding how structure and function influence biomolecular interactions; NanoTemper Technologies' Monolith X instrument, with its dual measurement modes Spectral Shift and TRIC, defines a new standard capable of handling delicate or low-abundance membrane proteins with precision, reliability, and ease-of-use.

Pairing the Monolith X instrument with Cube Biotech's NativeMP™ Technology offers a unified workflow that minimizes sample preparation while preserving the structural and functional integrity of membrane proteins. These innovative technologies enable a highly effective investigation of diverse binding interactions, such as protein-small molecule, protein-peptide, and protein-protein affinities, affirming this combinatorial approach as an indispensable tool for researchers in drug discovery, development, and biophysical analysis.

Highlights

No Need for Immobilization: Monolith X, when paired with the NativeMP™ Platform, ensures membrane proteins are stabilized within their natural lipid environment, maintaining their authentic structure and dynamics without the need for detergents. This approach eliminates the challenges of immobilization, such as steric hindrance or conformational changes, ensuring binding pockets remain accessible and unaltered.

Native-Like Environment Compatibility: Monolith X uses advanced Spectral Shift and TRIC technologies for precise binding measurements directly in solution. These techniques detect binding events through fluorescence changes rather than mass shifts, making them highly effective for large protein complexes. This approach ensures compatibility with native-like lipid or copolymer environments, enabling accurate analysis of membrane protein interactions under near-native conditions.

Small Sample Requirement: The Monolith X system requires minimal sample amounts, a significant advantage for membrane proteins that are often challenging to prepare and yield in limited quantities. This efficient use of resources makes it an ideal solution for studying rare or delicate targets, as demonstrated in complex interactions like NTCP and LHBsAg, where high analyte yields could otherwise pose purification challenges.

No Mass Disparity Sensitivity: Monolith X excels in detecting interactions across a wide range of molecular weights by leveraging fluorescence-based sensitivity. This capability allows it to resolve binding events between large protein complexes and small molecule analytes, such as the P2X4 receptor (150 kDa in its trimeric form with additional lipid mass) and 5-BDBD (355 Da). Even with extreme mass differences, Monolith X delivers precise and reliable results, overcoming limitations typically faced in mass-dependent detection systems.

Fast Turnover Times: Monolith X delivers rapid results, a measurement of a dilution series takes no longer than 6 minutes combining Spectral Shift and TRIC. This efficiency is ideal for studying tight binders or hydrophobic small molecules prone to aggregation in aqueous solutions. The shorter processing time improves data quality and increases the success rate of binding studies.

References

1. Baroja-Mazo A, Barbera-Cremades M, Pelegrin P. (2013). The participation of plasma membrane hemichannels to purinergic signaling. Biochim Biophys Acta, 1828:79-93.

2. Antonio LS, Stewart AP, Varanda WA, Edwardson JM. (2014). Identification of P2X2/P2X4/P2X6 heterotrimeric receptors using atomic force microscopy (AFM) imaging. FEBS Lett 588:2125-8.

3. Kaczmarek-Hajek K, Lorinczi E, Hausmann R, Nicke A. (2012). Molecular and functional properties of P2X receptorsrecent progress and persisting challenges. Purinergic Signal, 8:375-417.

4. Burnstock G, Kennedy C. (2011). P2X receptors in health and disease. Adv Pharmacol, 61:333-72.

5. Pankratov, Y. V., Lalo, U. V. & Krishtal, O. A. (2002). Role for P2X receptors in long-term potentiation. J. Neurosci., 2002;22:8363–-8369.

6. Tsuda, M. et al. (2003). P2X 4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature, 424, 778–-783.

7. Müller TD, Finan B, Bloom SR, D'Alessio D, Drucker DJ, Flatt PR, Fritsche A, Gribble F, Grill HJ, Habener JF, Holst JJ, Langhans W, Meier JJ, Nauck MA, Perez-Tilve D, Pocai A, Reimann F, Sandoval DA, Schwartz TW, Seeley RJ, Stemmer K, Tang-Christensen M, Woods SC, DiMarchi RD, Tschöp MH. Glucagon-like peptide 1 (GLP-1). Mol Metab. 2019 Dec;30:72-130. doi: 10.1016/j.molmet.2019.09.010. Epub 2019 Sep 30. PMID: 31767182; PMCID: PMC6812410.

8. Ferhatbegović L, Mršić D, Macić-Džanković A. The benefits of GLP1 receptors in cardiovascular diseases. Front Clin Diabetes Healthc. 2023 Dec 8;4:1293926. doi: 10.3389/fcdhc.2023.1293926. PMID: 38143794; PMCID: PMC10739421.

9. Chilton, R.J., Wyatt, J., Nandish, S., Oliveros, R.A., & Lujan, M. (2011). Cardiovascular comorbidities of type 2 diabetes mellitus: defining the potential of glucagonlike peptide-1-based therapies. The American journal of medicine, 124 1 Suppl, S35-53 .

10. Irwin, N., Flatt, P.R., Patterson, S., & Green, B.D. (2010). Insulin-releasing and metabolic effects of small molecule GLP-1 receptor agonist 6,7-dichloro-2-methylsulfonyl-3-N-tert-butylaminoquinoxaline. European journal of pharmacology, 628 1-3, 268-73 .

11. Huinan Zhang, Yunhan Liu, Liusiyuan Cheng, Xue Ma, Xiaoxing Luo, Exendin-4 induces a novel extended effect of ischemic tolerance via crosstalk with IGF-1R,Brain Research Bulletin, Volume 169, 2021, Pages 145-155, ISSN 0361-9230, https://doi.org/10.1016/j.brainresbull.2020.11.008.

12. Cheng, D., Yang, S., Zhao, X., & Wang, G. (2022). The Role of Glucagon-Like Peptide-1 Receptor Agonists (GLP-1 RA) in Diabetes-Related Neurodegenerative Diseases. Drug Design, Development and Therapy, 16, 665 - 684.

13.Pocai, A. (2023). G protein-coupled receptors and obesity. Frontiers in Endocrinology, 14.

14. Baggio, L.L., & Drucker, D.J. (2020). Glucagon-like peptide-1 receptor co-agonists for treating metabolic disease. Molecular Metabolism, 46.

15. Nauck, M.A., Quast, D.R., Wefers, J., & Meier, J.J. (2020). GLP-1 receptor agonists in the treatment of type 2 diabetes – state-of-the-art. Molecular Metabolism, 46.

16. Candido, R. (2021). GLP-1 receptor agonists: evolution and advancement in the treatment of type 2 diabetes mellitus. The Journal of AMD.

17. Huang, H., Huang, H., Chiou, W., Lin, L., Chen, J., Liu, H., Lai, Y., & Huang, C. (2021). Ergosterol peroxide inhibits HBV infection by inhibiting the binding of the pre-S1 domain of LHBsAg to NTCP. Antiviral research, 105184 .

18. Nkongolo, S., Ni, Y., Lempp, F.A., Kaufman, C., Lindner, T., Esser-Nobis, K., Lohmann, V., Mier, W., Mehrle, S., & Urban, S. (2014). Cyclosporin A inhibits hepatitis B and hepatitis D virus entry by cyclophilin-independent interference with the NTCP receptor. Journal of hepatology, 60 4, 723-31 .

19. Appelman, M.D., Chakraborty, A., Protzer, U., McKeating, J.A., & van de Graaf, S.F. (2017). N-Glycosylation of the Na+-Taurocholate Cotransporting Polypeptide (NTCP) Determines Its Trafficking and Stability and Is Required for Hepatitis B Virus Infection. PLoS ONE, 12.

20. Zakrzewicz, D., Leidolf, R., Kunz, S., Müller, S.F., Neubauer, A., Leiting, S., Goldmann, N., Lehmann, F., Glebe, D., & Geyer, J. (2022). Tyrosine 146 of the Human Na+/Taurocholate Cotransporting Polypeptide (NTCP) Is Essential for Its Hepatitis B Virus (HBV) Receptor Function and HBV Entry into Hepatocytes. Viruses, 14.

21. Ni, Y., Lempp, F.A., Mehrle, S., Nkongolo, S., Kaufman, C., Fälth, M., Stindt, J., Königer, C., Nassal, M., Kubitz, R., Sültmann, H., & Urban, S. (2014). Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology, 146 4, 1070-83.

22. Li, Y., Zhou, J., & Li, T. (2022). Regulation of the HBV Entry Receptor NTCP and its Potential in Hepatitis B Treatment. Frontiers in Molecular Biosciences, 9.

23. Calabria, Andrew C., et al. “GLP-1 Receptor Antagonist Exendin-(9-39) Elevates Fasting Blood Glucose Levels in Congenital Hyperinsulinism Owing to Inactivating Mutations in the ATP-Sensitive K+ Channel.” Diabetes, vol. 61, no. 10, Oct. 2012, pp. 2585–91, https://doi.org/10.2337/db12-0166.

24. Coddou, C., Sandoval, R., Hevia, M., & Stojilkovic, S. (2019). Characterization of the antagonist actions of 5-BDBD at the rat P2X4 receptor. Neuroscience Letters, 690, 219-224. https://doi.org/10.1016/j.neulet.2018.10.047.

25. Bidula, S., Nadzirin, I., Cominetti, M., Hickey, H., Cullum, S., Searcey, M., Schmid, R., & Fountain, S. (2021). Structural Basis of the Negative Allosteric Modulation of 5-BDBD at Human P2X4 Receptors. Molecular Pharmacology, 101, 33 - 44. https://doi.org/10.1124/molpharm.121.000402.