NativeMP™ Platform

Next Generation Copolymers for the Characterization of Membrane Proteins in Near-Native Conditions

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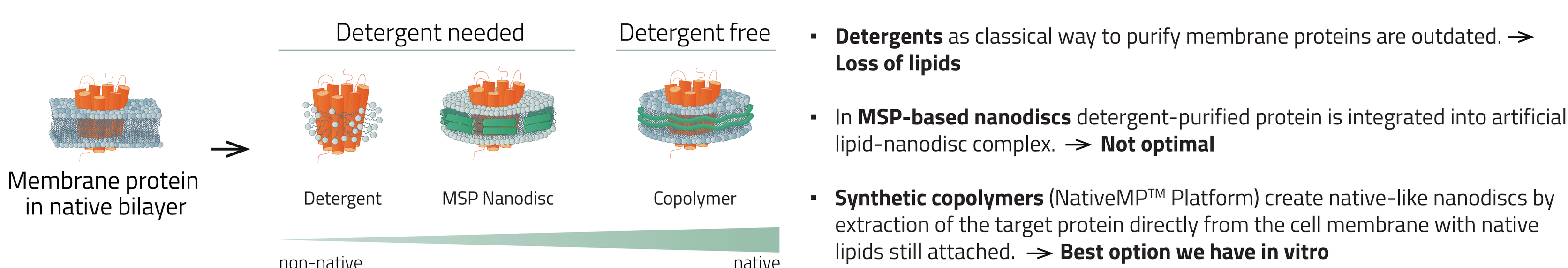
Abstract: The study of membrane proteins has been revolutionized by the development of next-generation copolymers, offering profound advancements in purification and structural characterization. Membrane proteins, particularly G protein-coupled receptors (GPCRs) and ion channels, are critical for cellular signaling and homeostasis, and represent approximately 60% of current drug targets. Research on these proteins is essential to combat a broad range of diseases, including cardiovascular disorders, neurological conditions, and cancers. Traditional detergents, while useful, often strip away essential lipids, leading to protein instability and loss of function. In contrast, copolymers utilized in Cube Biotech's unique NativeMP™ platform maintain a lipid-rich environment that preserves the native state of these proteins. We present a comprehensive workflow, from gene to structure, utilizing next-generation polymers for full-length membrane protein purification. We demonstrate the efficacy of this approach with a case study on an ion channel, showcasing the retention of functional lipids and the maintenance of protein activity throughout the purification process. The introduction highlights the pivotal role of lipids in membrane protein folding and function, as corroborated by studies from Dorr et al. (2016), Bersch et al. (2017), and Smirnova et al. (2020). Our methodology starts with gene cloning and expression in a suitable host, followed by solubilization with our NativeMP™ platform. These copolymers enable the extraction of membrane proteins in a lipid environment that closely mimics their native conditions. Subsequent steps include purification, functional assays, and structural analysis using cryo-electron microscopy (cryo-EM). Results reveal that proteins retain their functional integrity and are amenable to high-resolution structural determination. This approach represents a significant leap forward in membrane protein research, offering a viable alternative to detergent-based methods. By preserving the lipid milieu, next-generation copolymers facilitate the study of membrane proteins in a state that closely resembles their physiological context. This enables more accurate insights into their mechanisms and interactions, paving the way for advancements in drug discovery. Our findings underscore the potential of copolymer-based techniques to transform the landscape of membrane protein characterization and functional analysis, thereby enhancing our ability to develop targeted therapies for a wide range of diseases.

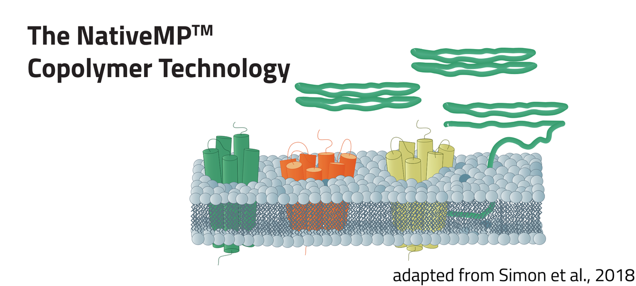
1. Introduction Part One

Stabilizing Membrane Proteins



- **Detergents** as classical way to purify membrane proteins are outdated. → **Loss of lipids**
- In **MSP-based nanodiscs** detergent-purified protein is integrated into artificial lipid-nanodisc complex. → **Not optimal**
- **Synthetic copolymers** (NativeMP™ Platform) create native-like nanodiscs by extraction of the target protein directly from the cell membrane with native lipids still attached. → **Best option we have in vitro**

The NativeMP™ Copolymer Technology



adapted from Simon et al., 2018

1. Introduction Part 2

Our Workflow

Significant cost/time reduction for cryo-EM
- Single symmetrical SEC peak of P2X4 in U-18

Sample use at higher temperature possible
- Temperature stability of P2X4 in U-18

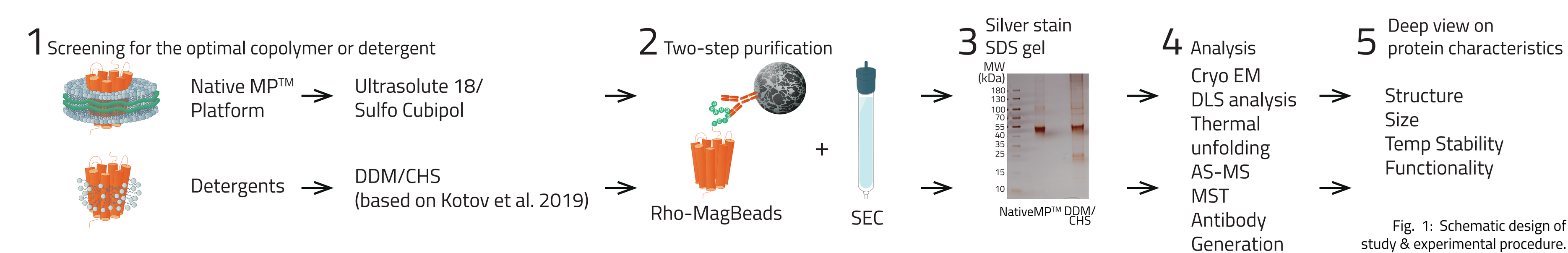


Fig. 1: Schematic design of study & experimental procedure.

2. Screening is Key

The NativeMP™ Platform vs Detergent

2A Working at 23 °C significantly boosts elution quantity (up to 200%) compared to 4 °C

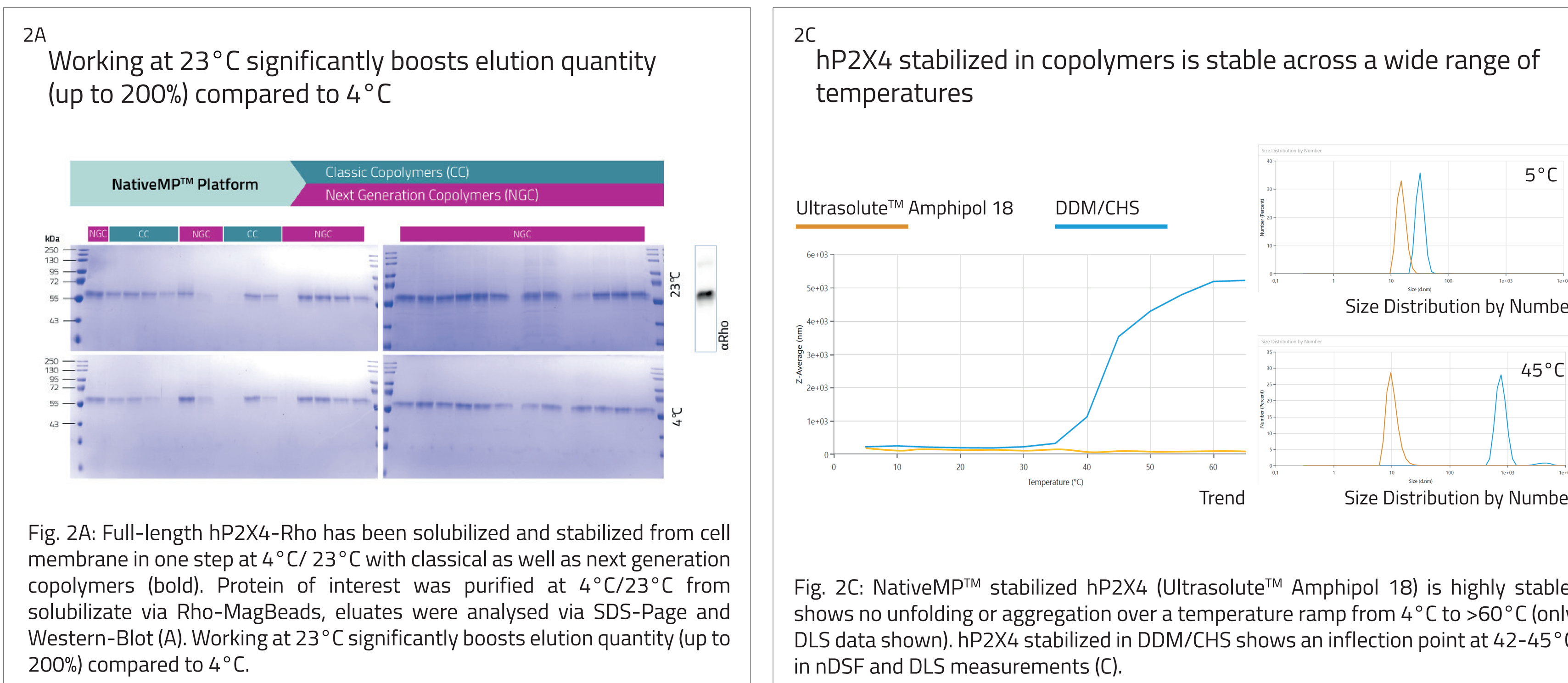
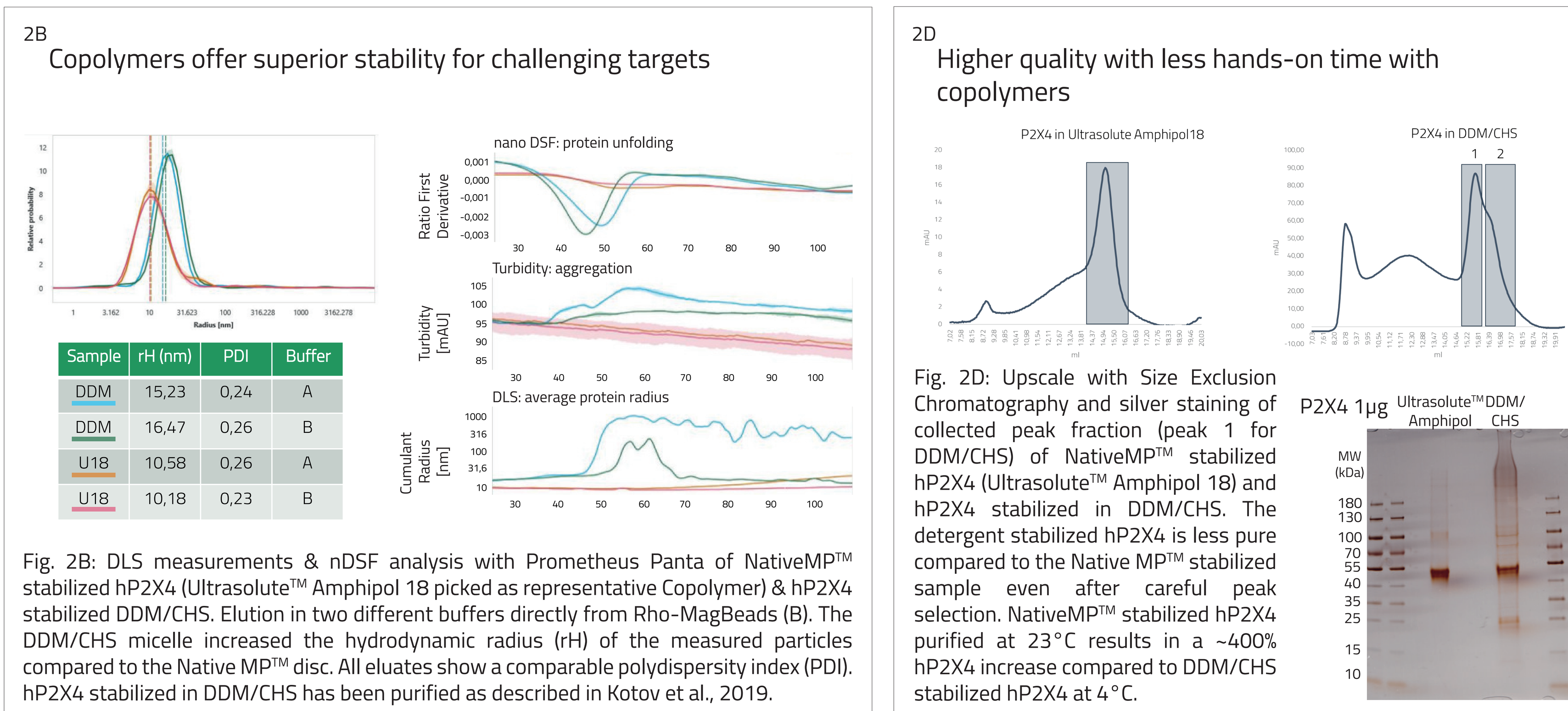


Fig. 2A: Full-length hP2X4-Rho has been solubilized and stabilized from cell membrane in one step at 4 °C/ 23 °C with classical as well as next generation copolymers (bold). Protein of interest was purified at 4 °C/23 °C from solubilizate via Rho-MagBeads, eluates were analysed via SDS-Page and Western-Blot (A). Working at 23 °C significantly boosts elution quantity (up to 200%) compared to 4 °C.

2C hP2X4 stabilized in copolymers is stable across a wide range of temperatures

Fig. 2C: NativeMP™ stabilized hP2X4 (Ultrasolute™ Amphipol 18) is highly stable, shows no unfolding or aggregation over a temperature ramp from 4 °C to >60 °C (only DLS data shown). hP2X4 stabilized in DDM/CHS shows an inflection point at 42-45 °C in nDSF and DLS measurements (C).

2B Copolymers offer superior stability for challenging targets



Sample	rH (nm)	PDI	Buffer
DDM	15,23	0,24	A
DDM	16,47	0,26	B
U18	10,58	0,26	A
U18	10,18	0,23	B

Fig. 2B: DLS measurements & nDSF analysis with Prometheus Panta of NativeMP™ stabilized hP2X4 (Ultrasolute™ Amphipol 18 picked as representative Copolymer) & hP2X4 stabilized DDM/CHS. Elution in two different buffers directly from Rho-MagBeads (B). The DDM/CHS micelle increased the hydrodynamic radius (rH) of the measured particles compared to the Native MP™ disc. All eluates show a comparable polydispersity index (PDI). hP2X4 stabilized in DDM/CHS has been purified as described in Kotov et al., 2019.

2D Higher quality with less hands-on time with copolymers

Fig. 2D: Upscale with Size Exclusion Chromatography and silver staining of collected peak fraction (peak 1 for DDM/CHS) of NativeMP™ stabilized hP2X4 (Ultrasolute™ Amphipol 18) and hP2X4 stabilized in DDM/CHS. The detergent stabilized hP2X4 is less pure compared to the Native MP™ stabilized sample even after careful peak selection. NativeMP™ stabilized hP2X4 purified at 23 °C results in a ~400% hP2X4 increase compared to DDM/CHS stabilized hP2X4 at 4 °C.

3. Making the Impossible Possible

Copolymer Stabilized P2X4 Enables Antibody Generation

ProteoGenix

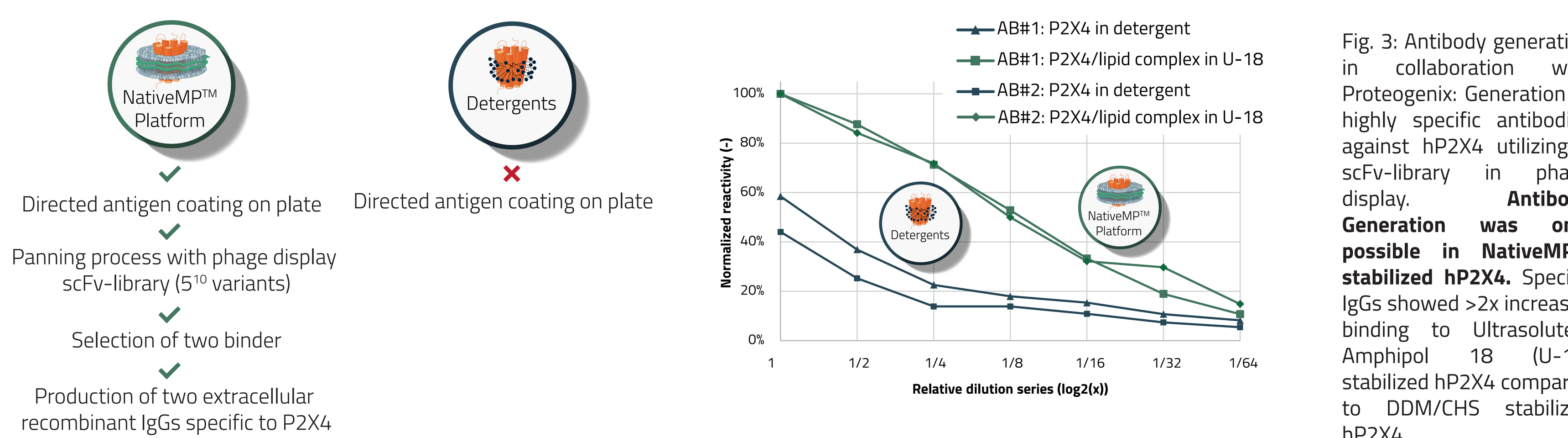
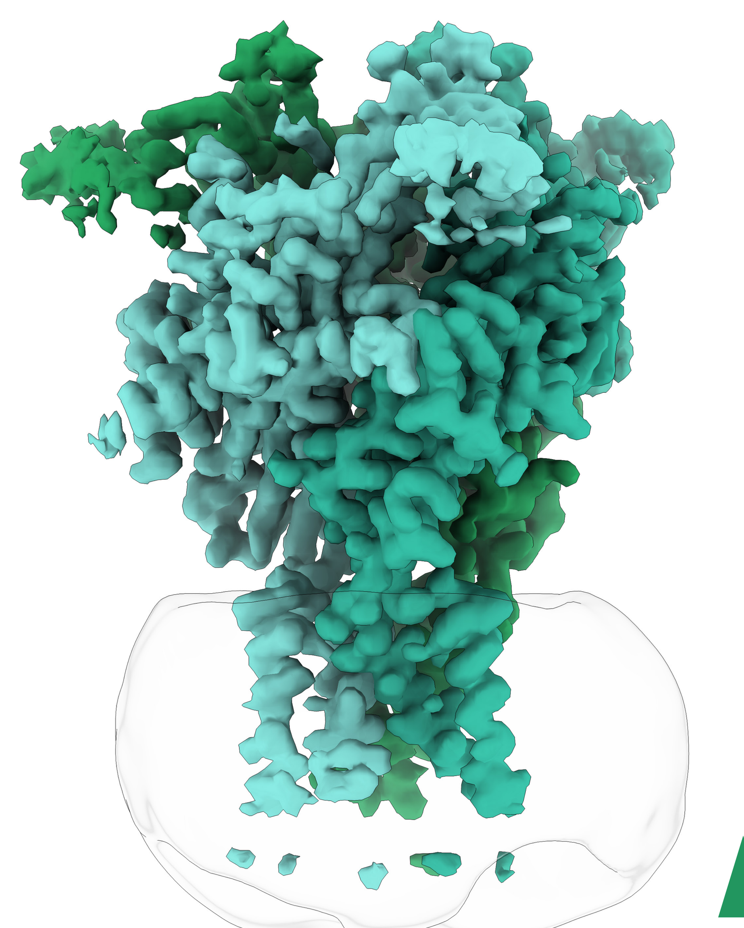


Fig. 3: Antibody generation in collaboration with Proteogenix: Generation of highly specific antibodies against hP2X4 utilizing a scFv-library in phage display. **Antibody Generation was only possible in NativeMP™ stabilized hP2X4.** Specific IgGs showed >2x increased binding to Ultrasolute™ Amphipol 18 (U-18) stabilized hP2X4 compared to DDM/CHS stabilized hP2X4.

Finding The Perfect Match

hP2x4 Full Length Structure



Cube Biotech

6. Finding The Perfect Match

Structure Determination

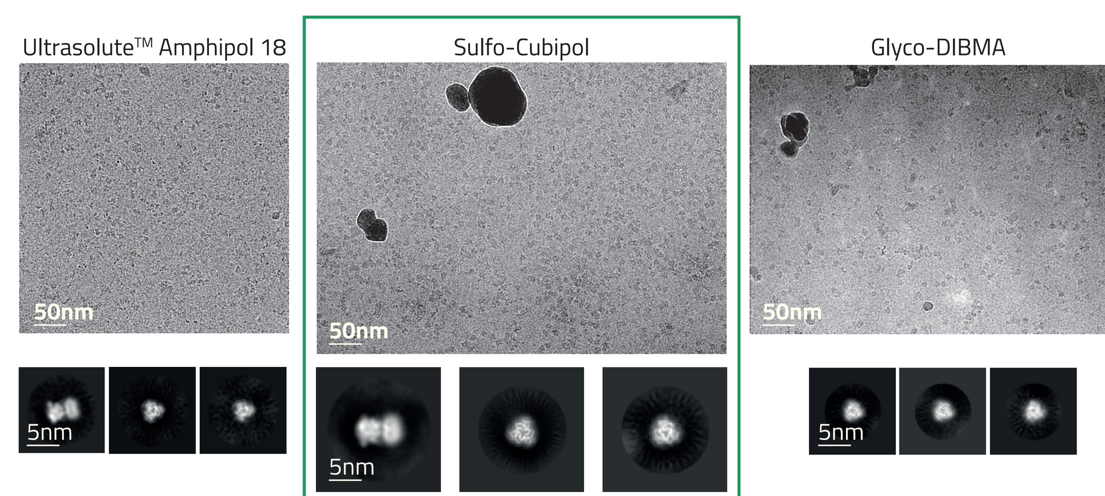


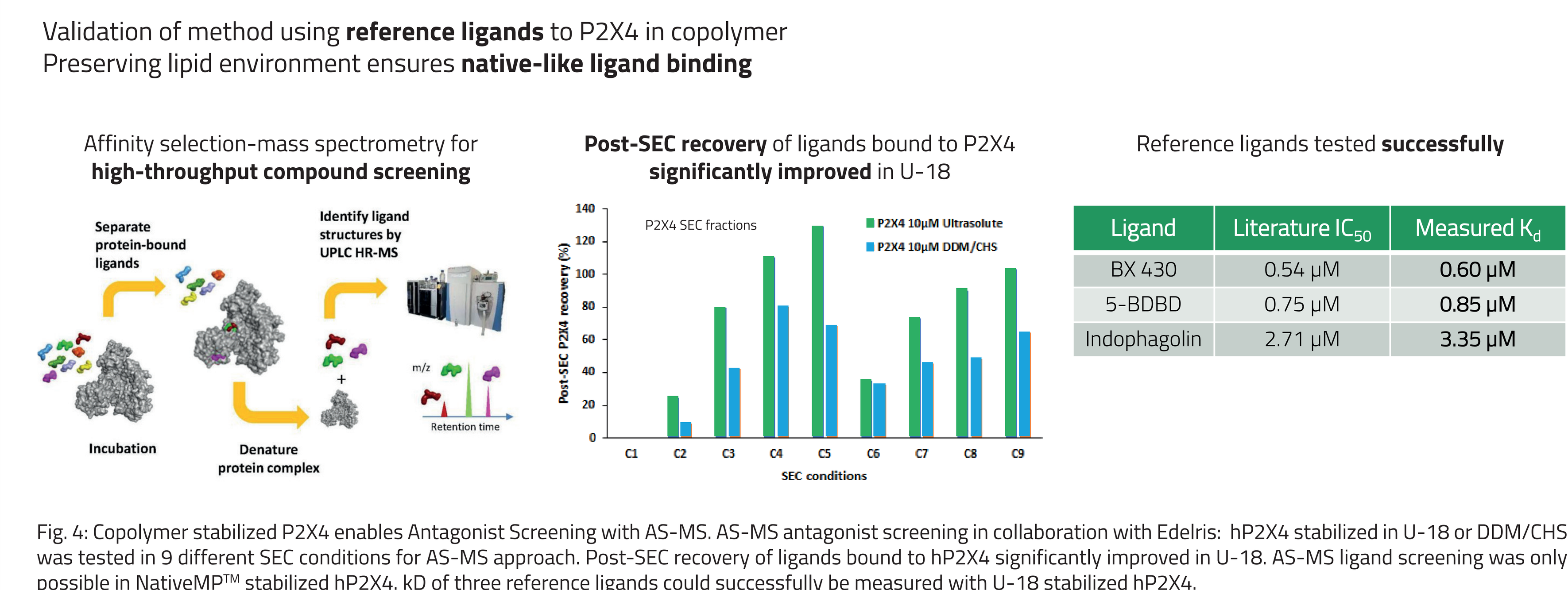
Fig. 6: Careful copolymer selection and an extensive screening process is the key to success when trying to obtain optimal results in cryo-EM. Sulfo-Cubipol produces an optimal particle orientation distribution for hP2X4. **Early refinement cycles already produce a reconstruction of < 3.5 Å global resolution.**

4. Yet Again Impossible in Detergent – Possible in NativeMP™

First Ever Ion-Channel Ligand Screened in AS-MS

edelris

Validation of method using **reference ligands** to P2X4 in copolymer
Preserving lipid environment ensures **native-like ligand binding**



Ligand	Literature IC_{50}	Measured K_d
BX 430	0.54 µM	0.60 µM
5-BDBD	0.75 µM	0.85 µM
Indophagolin	2.71 µM	3.35 µM

Fig. 4: Copolymer stabilized P2X4 enables Antagonist Screening with AS-MS. AS-MS antagonist screening in collaboration with Edelris: hP2X4 stabilized in U-18 or DDM/CHS was tested in 9 different SEC conditions for AS-MS approach. Post-SEC recovery of ligands bound to hP2X4 significantly improved in U-18. AS-MS ligand screening was only possible in NativeMP™ stabilized hP2X4. kD of three reference ligands could successfully be measured with U-18 stabilized hP2X4.

5. Reproduction in Method Variety

Copolymer enables Antagonist Screening with MST/ Spectral Shift

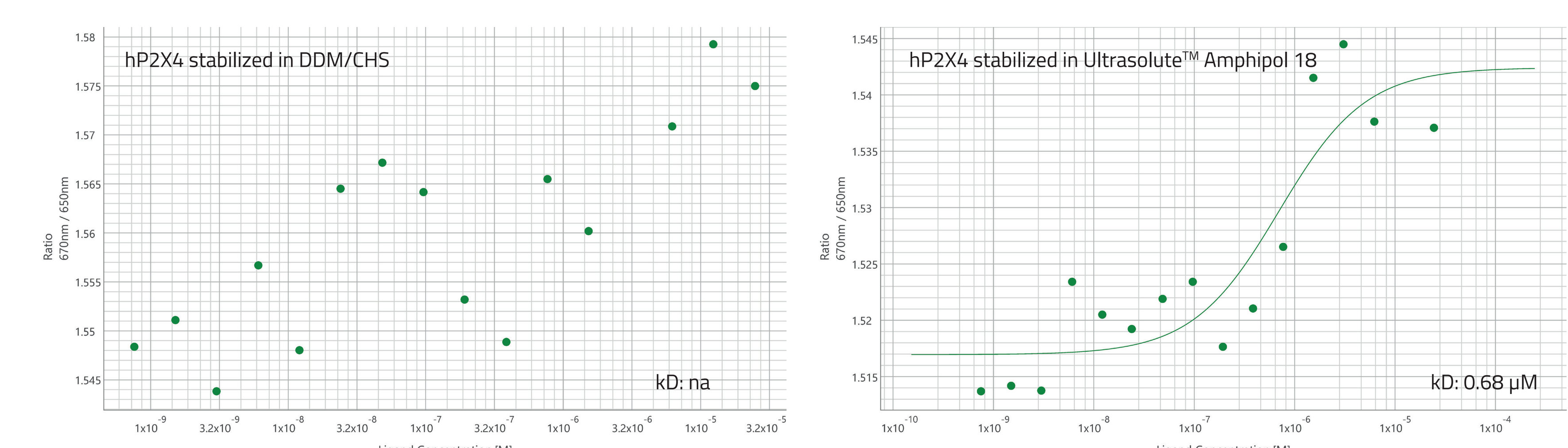


Fig. 5: hP2X4 stabilized in U-18 or DDM/CHS was labeled with a 647-NHS dye and mixed with increasing amounts of 5-BDBD. The binding affinity was measured in solution using a Nanotemper Monolith and standard capillaries after binding check and buffer optimization. A kD measurement was only possible when hP2X4 was stabilized in synthetic copolymer (U-18, kD: 0.68 µM). DDM/CHS stabilized hP2X4 showed no measureable kD when mixed with 5-BDBD.

7. Conclusion

Universal Applicability Beyond hP2X4

Copolymers have a broad range of advantages compared to classic detergents. The NativeMP™ Platform is the way to go if you want to purify active, stable and correctly folded membrane proteins in their native lipid environment. We show that the NativeMP™ Platform beats detergent in hands-on time, efficiency, temperature stability, functionality, storability, ligand interaction and antibody generation. Copolymers are the future of membrane protein science and the drug targeting market.

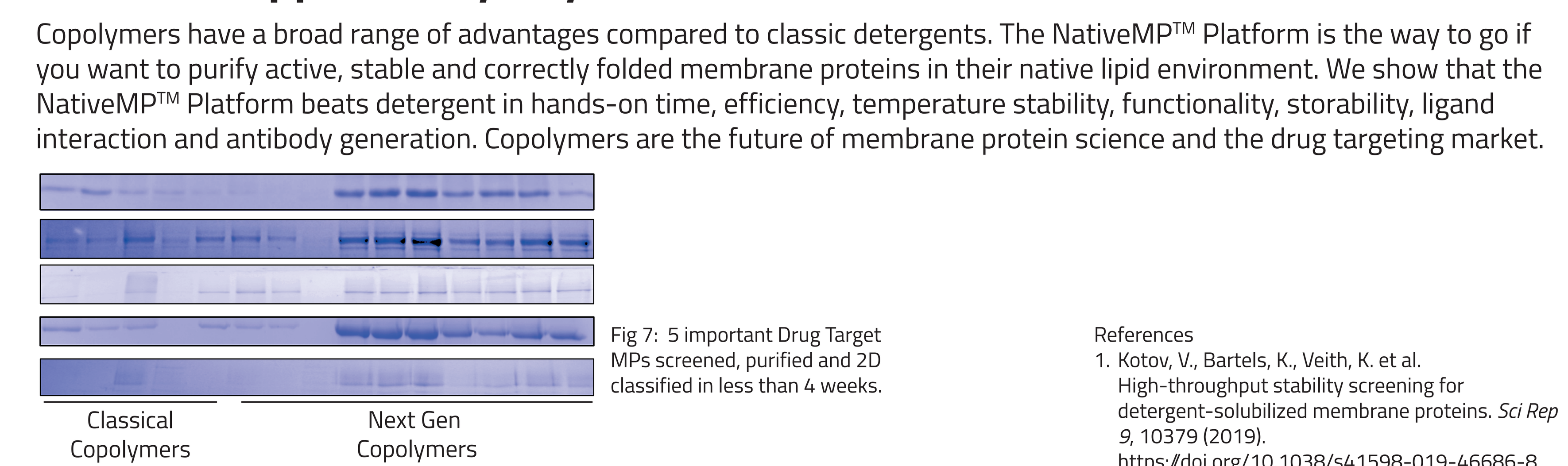


Fig 7: 5 important Drug Target MPs screened, purified and 2D classified in less than 4 weeks.

References
1. Kotov, V., Bartels, K., Veith, K. et al. High-throughput stability screening for detergent-solubilized membrane proteins. *Sci Rep 9*, 10379 (2019). https://doi.org/10.1038/s41598-019-46686-8