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WHITE PAPER

Cryo-EM structure of ion channel pannexin 1 using GeneArt and Salipro DirectMX platforms

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Introduction

Cryo-electron microscopy (cryo-EM) is rapidly evolving to be the primary tool for the structure determination of membrane proteins[1,2]. Ion channels are particularly prominent examples since they are important drug targets and conducive in terms of molecular weight. Like all membrane proteins, production of structure-grade recombinant ion channel proteins requires experience and expertise, often making this step the bottleneck of the workflow. We asked whether it would be possible to leverage GeneArt™ services from Thermo Fisher Scientific with Salipro Biotech's expertise and the Salipro® platform technology for membrane proteins to generate high-quality pannexin 1 (PANX1) ion channel protein for structure elucidation by cryo-EM (Fig. 1). In this white paper, we present the three stages of our approach – protein expression, generation of purified Salipro®-PANX1, and structure determination (Fig. 2).

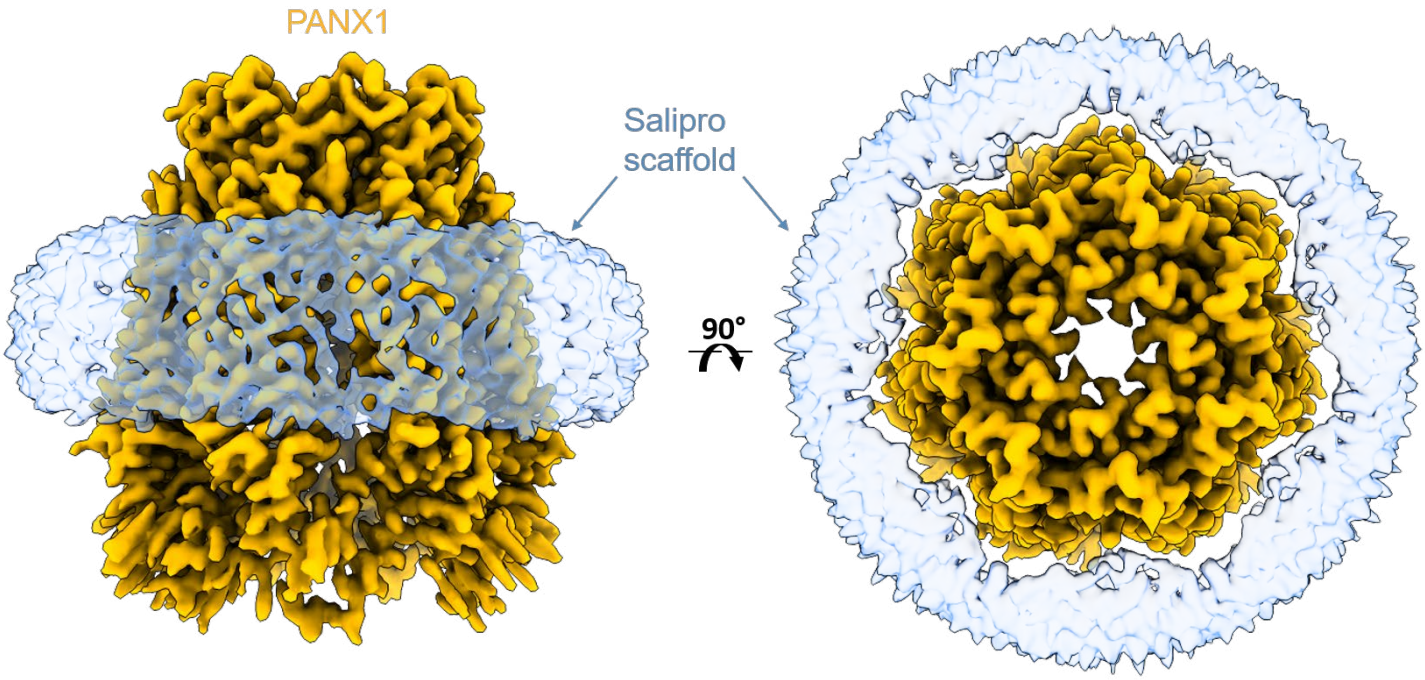

Figure 1. Cryo-EM reconstruction of Salipro-PANX1 at 3.2 Å. The ion channel is displayed in yellow while the Salipro-lipid disk is in transparent blue in side view (left) and top view (right).

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GeneArt	Salipro Biotech	Cryo-EM
• Gene design • Gene synthesis • Expression	• Screening • One-step reconstruction • Production analysis	• Specimen preparation • Data collection • Image reconstruction

Figure 2. A gene-to-structure pipeline to generate high-resolution cryo-EM structures of membrane proteins.

Thermo Fisher GeneArt

The GeneArt platform comprises a Gene-to-Protein service that only requires a protein sequence and covers every step from gene synthesis to protein purification. The service involves Thermo Fisher's proprietary mammalian and insect expression systems - Expi293™, ExpiCHO™, or ExpiSf9™. These cell lines form an excellent basis for membrane protein overexpression. Since GeneArt focuses on the purification of soluble intracellular and secreted proteins, we collaborated with Salipro Biotech for expertise in membrane proteins.

Salipro Biotech

Membrane proteins are important drug targets (e.g. GPCRs, ion channels), yet are notoriously difficult to work with. These proteins are embedded in the lipid membrane, however, once extracted from their natural lipid environment, membrane proteins are often unstable and challenging to handle. To address this problem, Salipro Biotech developed a proprietary nano-membrane platform technology (Salipro®) that stabilizes all types of integral membrane proteins in their native forms in a lipid environment[3], enabling them to be employed in drug discovery programs for therapeutic antibodies, small molecule drugs, and structure-based drug design (Fig. 3). With the DirectMX® methodology, Salipro Biotech has developed a one-step approach that allows for the direct purification and reconstitution of membrane proteins directly from crude cells[4].

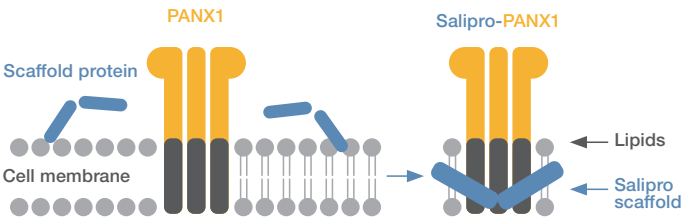

Figure 3. Schematic illustration of the reconstitution of membrane proteins into Salipro® particles.

Thermo Fisher Scientific cryo-EM

Life scientists interested in understanding proteins and protein complexes can use cryo-EM to determine their 3D structures without the need for crystals. This allows researchers to investigate protein function, their role in disease, and to design drugs with high precision. Cryo-EM has become the go-to method for challenging drug targets, particularly membrane proteins.

Ion channel PANX1

Our goal was to determine the cryo-EM structure of the PANX1 ion channel using a GeneArt-to-Salipro setup (Fig. 2). PANX1 is widely expressed throughout the body, mediating ATP export as a key function[5]. PANX1 holds promise as drug target for the treatment of various pathologies including inflammation, neuropathic pain, and addiction. In addition, PANX1 has been implicated as an off-target with numerous small molecule drugs. One such example is the antimalarial drug mefloquine, its binding to PANX1 has been linked to neurological side-effects[5]. To fully understand and exploit the biology of PANX1, highly selective and high affinity binders need to be developed. Several recent cryo-EM studies[6-10] have started to pave the way to discover new compounds with the required specificity by structure-based drug design (SBDD). Since many drug candidates will be evaluated in mouse models, we demonstrate here the expression, purification, and structure determination of murine PANX1 (Fig. 2).

Cryo-EM structure determination of pannexin 1

GeneArt gene synthesis and protein expression

Gene synthesis started with the electronic protein sequence of unmodified mouse PANX1 (Acc. No. AAH49074.1), fused with a C-terminal 3C protease cleavage site, GFP and a Strep tag (PANX1-3C-GFP-StrepII). The synthetic gene was designed for expression optimization in mammalian cells using the GeneOptimizer™ algorithm[11] and produced *de novo* through gene synthesis in vector pcDNA3.4. Gibco Expi293 cells were transiently transfected with this construct and cells corresponding to 5 liter cell culture were harvested after 3 days incubation. Cell pellets were sent to Salipro Biotech for the generation of Salipro®-PANX1.

Generation of purified Salipro®-PANX1

Membrane protein reconstitution is achieved by the Salipro® scaffold protein, which self-assembles into disc-like nanomembranes comprised of endogenous lipids and membrane proteins (Fig. 3). One of the advantages of the Salipro platform is that the scaffold flexibly adapts to the transmembrane regions of the drug target, making it possible to reconstitute membrane proteins independent of size, oligomeric state or shape in a direct setup from cell pellets.

Parts of the cell pellet was resuspended in reconstitution buffer, Salipro® scaffold protein was added for direct membrane extraction (DirectMX®) and Salipro®-PANX1 purified via Strep-tag. The Salipro-PANX1-3C-GFP-Strep loaded affinity resin was further incubated with PreScission protease and eluted Salipro-PANX1 nanoparticles were concentrated using ultra centrifugal filters. The sample was then subjected to Size Exclusion Chromatography (SEC) equilibrated with HN buffer (50 mM HEPES pH 7.5, 150 mM NaCl) and the Salipro-PANX1 containing SEC fractions were pooled, concentrated to 5 mg/mL, flash frozen and shipped for cryo-EM preparation.

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Cryo-EM sample preparation, data collection, and processing

Salipro-PANX1 sample was applied to glow-discharged Quantifoil R1.2/1.3 grids, blotted for 10 s using blot force 20 and plunge frozen into liquid ethane using Vitrobot Mark IV. Note that right before plunge freezing, 0.005% (w/v) fluorinated Fos-Choline 8 was added to the sample to overcome a preferred orientation. The data was collected on a Thermo Scientific™ Krios G4™ Cryo Transmission Electron Microscope (Cryo-TEM) equipped with Selectris X Imaging Filter and Falcon 4™ Direct Electron Detector camera operated in Electron-Event representation (EER) mode.

Data processing was performed in Relion single particle analysis suite[12]. After motion- and CTF-correction, ~1.1M particles were picked from 7339 micrographs. Following 2D and 3D classification, the best 3D class consisting of 108,268 particles was subjected to CTF Refinement, 3D refinement and post-processing, yielding an overall resolution of 3.2 Å (Fig. 1). Data acquisition and analysis parameters can be found in Table 1.

Table 1. Parameters used for cryo-EM data acquisition and analysis.

Data acquisition and processing parameters	
Grid	Quantifoil R1.2/1.3
Camera	Selectris X-Falcon 4
Slit width (eV)	10
Nominal magnification	165,000x
Pixel size (Å)	0.75
Dose rate (e-/pix/sec)	5.4
Exposure time (sec)	4.16
Total dose (e-/Å^2)	40.24
Fractionation	EER
Autofocus	After centering
Hole centering	AFIS (6 µm image shift)
Delay after stage shift (s)	5
Number of images	7339
Total no of particles picked	1,104,121
Particles in the final reconstruction	108,268

Discussion

In this case study, we demonstrate a setup where the expertise of Thermo Fisher Scientific and Salipro Biotech can be leveraged for the structural determination of membrane protein drug targets. Combining GeneArt and the Salipro® platform technologies for membrane proteins can therefore help overcome one of the biggest barriers of the cryo-EM workflow – obtaining high-quality membrane proteins of sufficient quality (Fig. 2). This protein for cryo-EM setup not only provides a means for SBDD but is also a powerful tool to validate the structural integrity of the protein, which is essential for a wide range of downstream workflows including the development of therapeutic antibodies. In summary, we believe that our solutions can in combination, or each separately bring the benefits of cryo-EM SBDD to pharma and biotech companies of all sizes.

Acknowledgments

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