Integral Membrane Proteins for Biophysics and Cryo-EM applications – Case Study of the Ion Channel TrpML3

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Introduction

- Integral membrane proteins are a large group of therapeutic targets in a variety of disease areas that remain difficult to investigate especially by biophysical and structural approaches
- TrpML3 is a homotetrameric ion channel (240 kDa) implicated in autophagy
- We showcase the extraction and purification of recombinant TrpML3 and the subsequent structural (CryoEM) and biophysical analysis of small molecule ligand binding.

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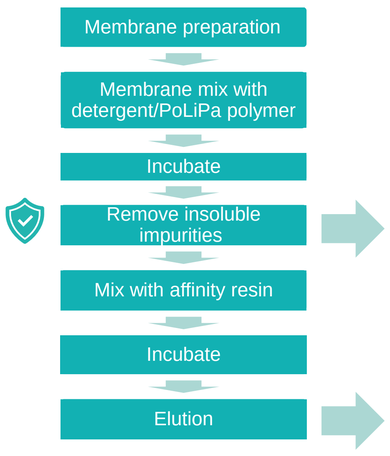
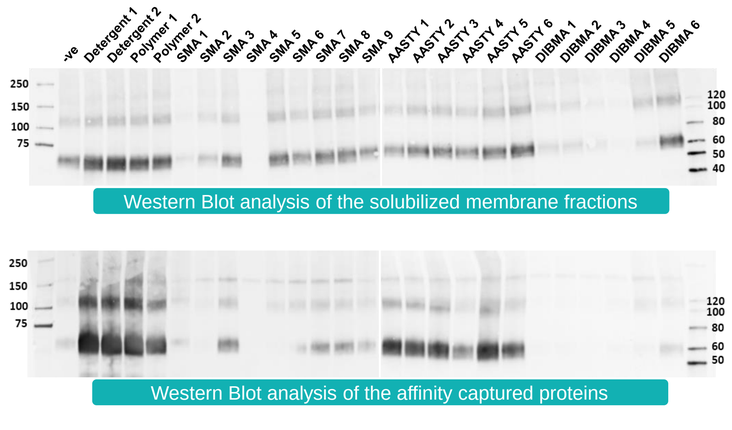
TrpML3 Detergent and Polymer Screening

Full-length TrpML3 was expressed in Expi293™ cells and the membrane fraction was extracted.

A membrane solubilisation screen with a range of detergents and amphipathic polymers was undertaken to identify effective solubilization conditions. Amphipathic polymers, such as SMA, DIBMA, AASTY, directly insert into cell membranes and spontaneously form nanodisc structures of native membrane lipids encapsulated by polymer. This allows membrane proteins to be extracted without removing them from the native lipid environment, which is beneficial for many downstream applications.

Solubilisation does not always correlate with tag accessibility, so further affinity capture and buffer optimisation is required to determine the optimal conditions for purification





Western Blot analysis of the solubilized membrane fractions

Western Blot analysis of the affinity captured proteins

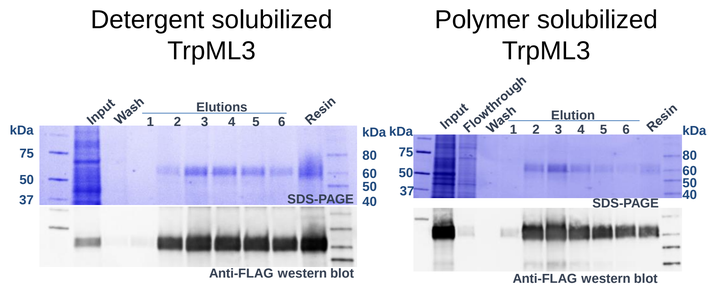
TrpML3 Protein Production

TrpML3 was extracted from the membranes using the LMNG detergent or the selected polymer from the solubilization screen and was affinity purified. Purity and yield was confirmed by SDS-PAGE and anti-Flag western blot.

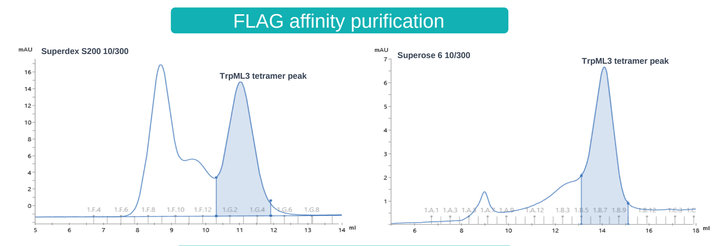
Size exclusion chromatography was used to isolate TrpML3 tetramers. From both preparations, TrpML3 eluted in a symmetric peak.

DLS analysis confirmed a single monodisperse population

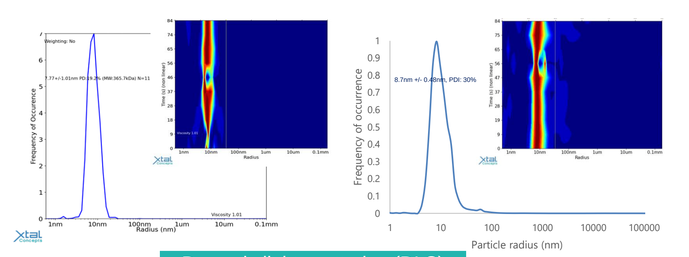
The yields are comparable between both preparations. After a two-step purification, a purity of > 90% was achieved and the obtained protein was suitable for downstream applications



FLAG affinity purification



Size exclusion chromatography



Dynamic light scattering (DLS)

TrpML3 Ligand Binding Confirmed by GCI

Membrane proteins are notoriously challenging to study in cell-free systems, especially so in biophysical surface capture methods such as Surface Plasmon Resonance (SPR) and Grating Coupled Interferometry (GCI). This is because of the difficulties in immobilizing large membrane proteins on biosensors, maintaining protein stability and the small binding signals obtained for small molecules.

The Creoptix WAVE instrument utilizes the proprietary waveRAPID® mode. Using waveRAPID®, one analyte concentration is pulsed repeatedly with increasing duration. The observed binding curve is a response to the time-dependent concentration input of the injected analyte. RAPID allows screening to be run 10x faster than traditional kinetic methods, saving time on both preparation and actual run time.

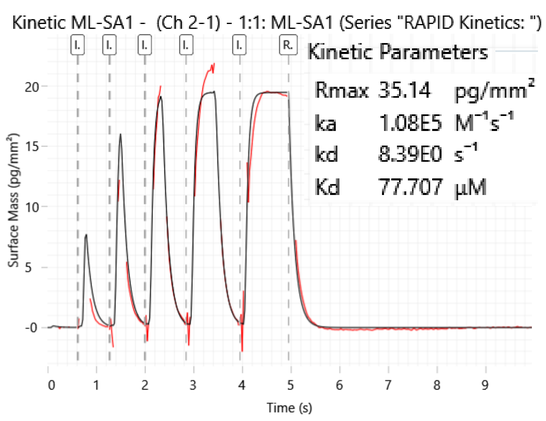
TrpML3 (240 kDa) was immobilized to a PCH-NTA chip via its His-tag. LMNG was included in the buffer to maintain the correct folding of the protein. Control compounds were tested for binding across this surface. The waveRAPID® mode was used to capture the kinetics of the ligand binding.

Compound	K_D (µM)
SF-22	16.9
ML-SA1	77.7
SN2	164.0
17 β - Estradiol	169.8
MK6-83	475.1

Results table of control compounds binding to TRPML3

All of the compounds tested showed binding in the micromolar range. Apart from MK6-83 the rank order of the K_Ds is the same as seen with IC_{50}s in literature. This demonstrates that the TRPML3 has been captured and maintained in its correctly folded state.

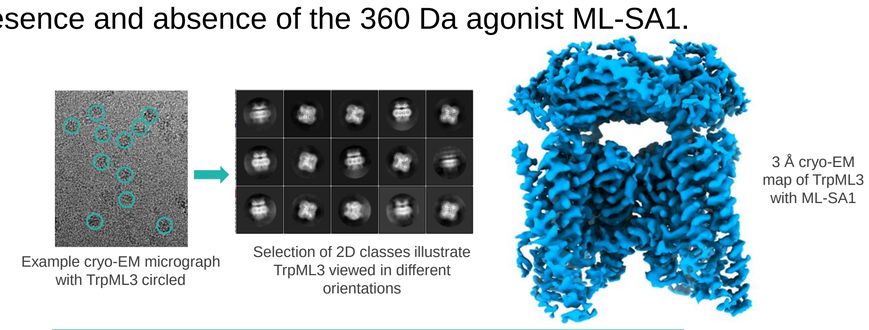
GCI was successfully able to capture detergent solubilized TrpML3 in its native conformation. This was demonstrated by the binding of control compounds to TrpML3, and the determination of their kinetics, despite its large size.



ML-SA1 binding to TRPML3 using WaveRAPID on the GCI

TrpML3 Ligand Binding Confirmed by Cryo-EM

Domainex has determined cryo-EM structures of TrpML3 solubilized in detergent LMNG, in the presence and absence of the 360 Da agonist ML-SA1.

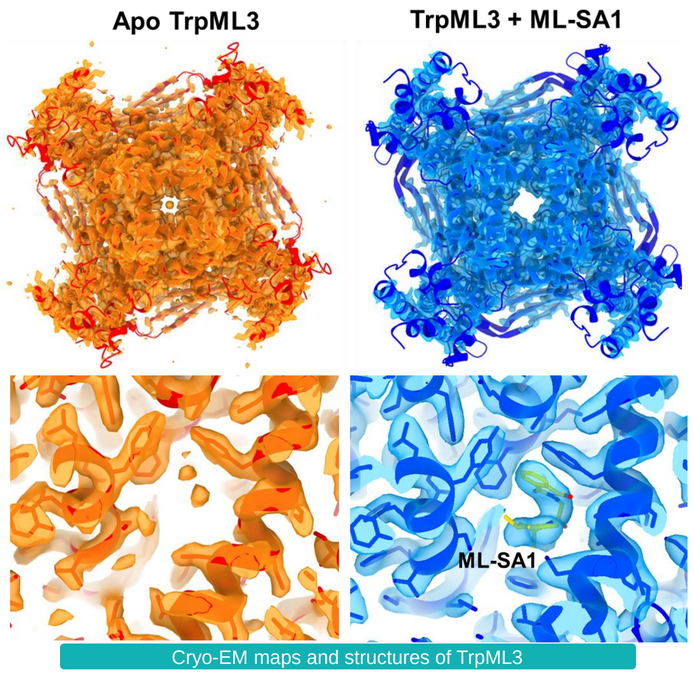


Cryo-EM analysis of TrpML3 flow chart

Cryo-EM grids were prepared using a Vitrobot (ThermoFisher) and analyzed on a 300KeV Titan Krios microscope with a K3 detector (ThermoFisher). The datasets were processed with RELION-4, yielding a 2.7 Å resolution map for apo TrpML3 and 3.0 Å resolution map for TrpML3 in complex with ML-SA1.

The apo TrpML3 was in a closed and inactive conformation, while in the presence of agonist ML-SA1, the channel was open. The resultant helical rearrangement between open and closed can be observed even at relatively low resolution.

Comparison of the high-resolution maps identified the ML-SA1 binding site, including side chain interactions leading to TrpML3 activation. The resultant TrpML3 structures have enabled computer-aided drug design (CADD) and virtual screening.



Cryo-EM maps and structures of TrpML3

The cryo-EM maps of TrpML3 are of suitable quality to model bound ligands and protein side chain conformations confidently. The 3D structures allow *ab-initio* drug design.

Services/Contact

If you would like to speak with us about TrpML3 or drug discovery services, please contact:
enquiries@domainex.co.uk

domainex.co.uk

Conclusions

- Ion channel TrpML3 was successfully purified using detergents or polymer-encapsulated nanodiscs.
- Ligand binding to LMNG purified TrpML3 was successfully confirmed by Grating-Coupled Interferometry (GCI) and Cryo-EM.
- Computational chemistry assessment confirms that the generated structures are suitable for Structure Based Drug design (SBDD).