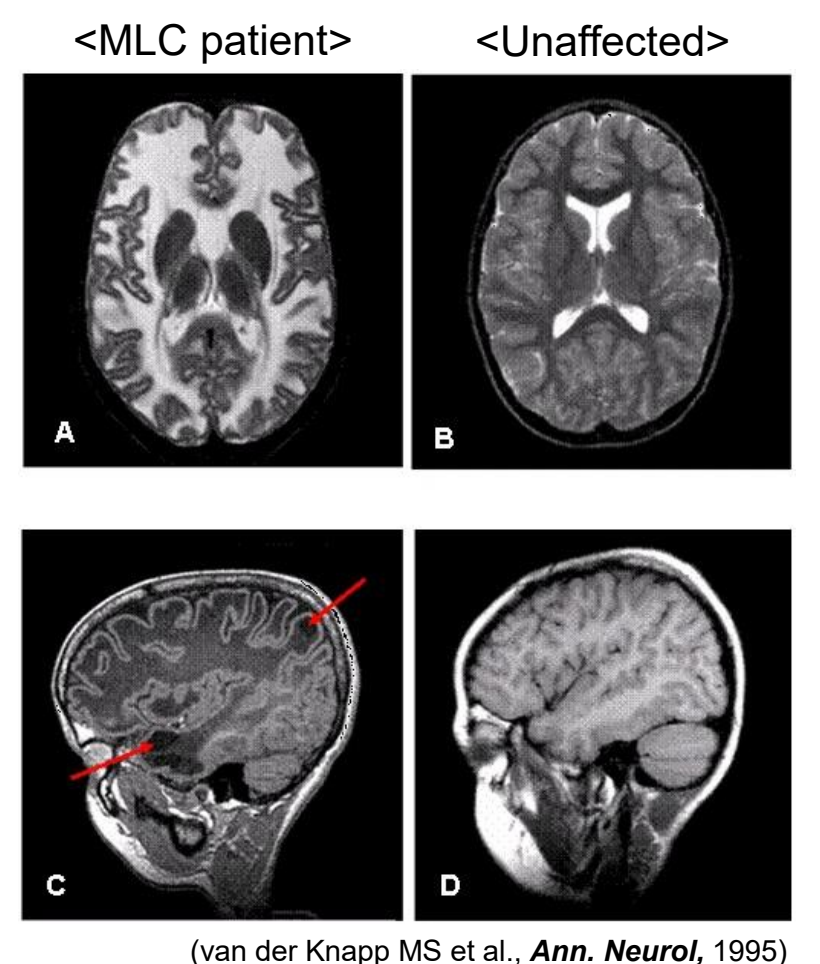


MLC1 is a membrane protein with eight transmembrane domains and is mainly expressed in astrocytes (Hwang J et al., *Open Biology*, 2021). Genetic mutants on Mlc1 are associated with an early-onset rare genetic neurological disease called Megalencephalic Leukoencephalopathy with Subcortical Cysts (MLC), which leads to progressive neurodegeneration. MLC typically presents with infantile macrocephaly, followed by progressive gait disturbances and cognitive impairment. However, the structure and function of MLC1 have not been fully understood.

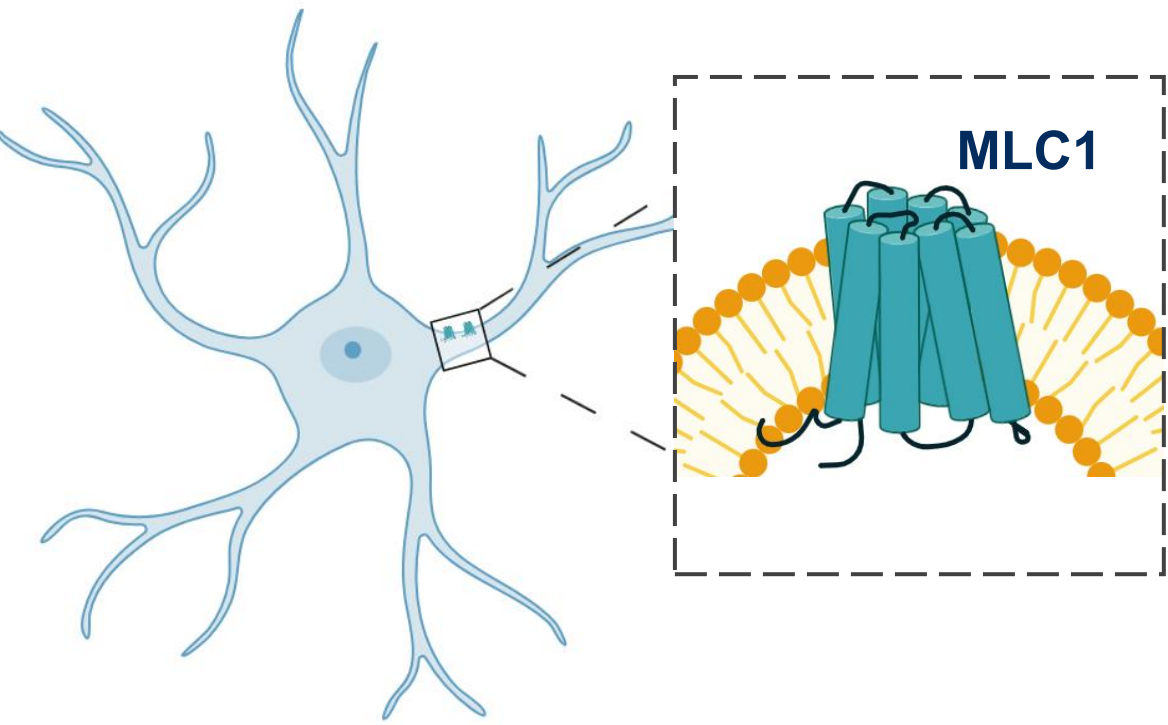
Thus, we aimed to determine a 3D-structure of human MLC1 protein using cryo-EM technique. We successfully expressed MLC1 proteins, in HEK293S GnTI⁻ cells and purified them for cryo-EM microscopic imaging. We have obtained ~3.3 Å dataset, though the orientation preference in the EM-grid hinder fine structural determination. Currently, we have been screening various conditions to identify an optimal condition for overcoming orientation preference. The addition of 0.0005~0.001% CTAB to the MLC1 in LMNG micelle showed improved cryograph. In addition, reconstitution of MLC1 protein into PE/PC/PG lipid nanodisc with 1E3 belt protein increase orientation diversity.

MLC, Megalencephalic Leukoencephalopathy with subcortical Cysts

- **Early childhood** onset with progressive neurological decline, Prevalence: ~1/500,000^{UK}, ~1/1,000,000^{Japan}.
- Caused by **MLC1 mutations (75%)** and **GlialCAM mutations (20%)** account for genetic etiology of MLC disease.
- Macrocephaly (enlarged brain), diffuse white matter swelling, subcortical cysts, myelin vacuolation and atrophy.
- Slow deterioration of motor functions, with **ataxia** (difficulty coordinating movements) and **spasticity** (stiffness of muscles and muscle spasms), **seizures** (well-controlled with medication).
- No direct treatment or cure for MLC.



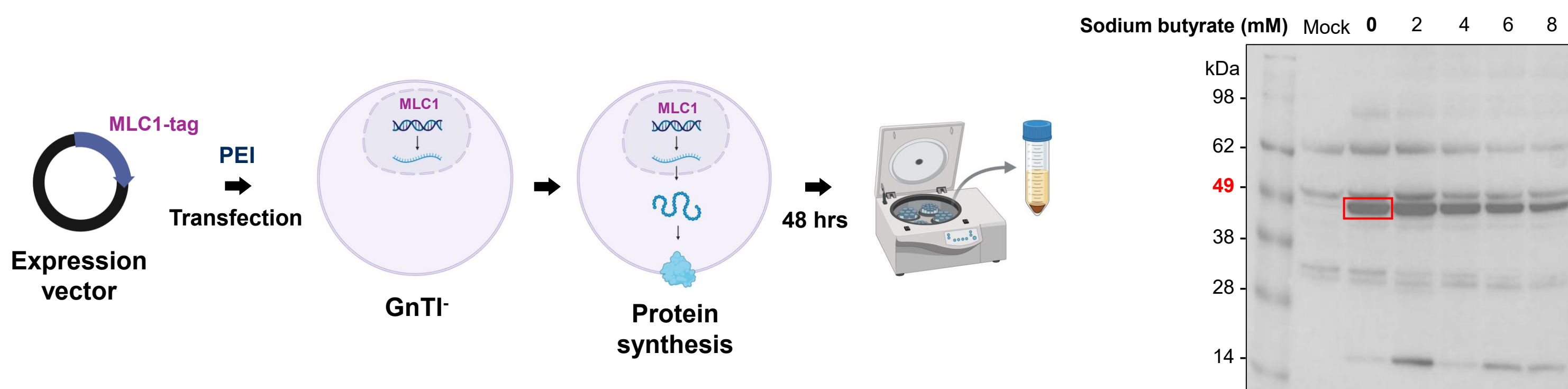
MLC1



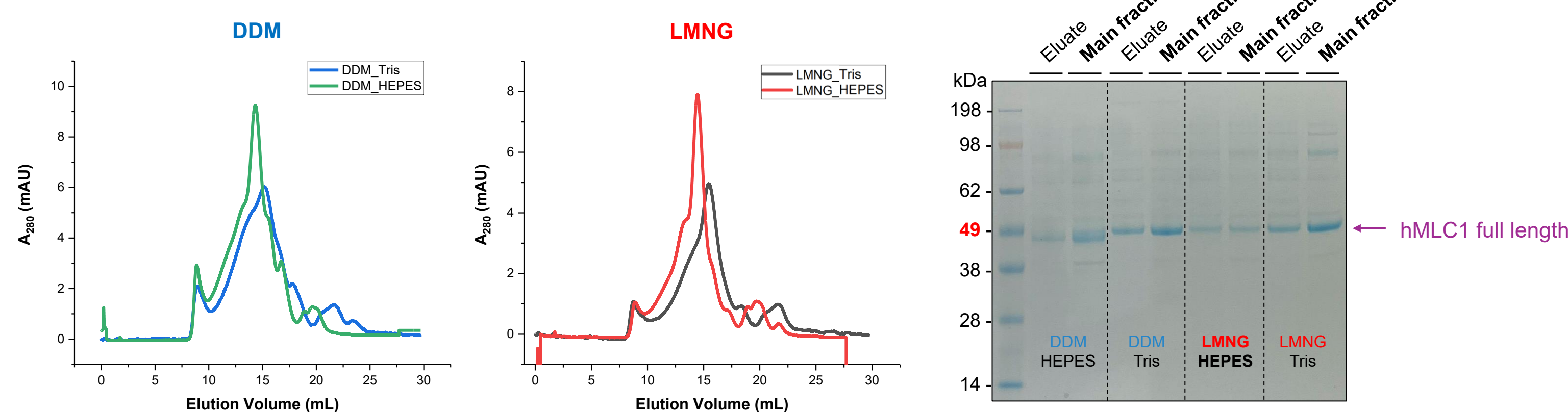
- **Eight-transmembrane protein** (~41kDa, human MLC1) (Hwang J et al., *Open Biology*, 2021).
- Evolutionary well conserved in the vertebrate animals.
- Mainly expressed in the **astrocyte end feet** and **astrocyte-astrocyte junction** (Estevez R et al., *Eur. J. Med. Genet.*, 2018).
- Functionally and Physically linked to CLC-2 channel and GlialCAM.
- Structurally and functionally unique: No homology with other membrane proteins, No known direct functions.

Optimization of MLC1 purification

I. Expression in mammalian cell line

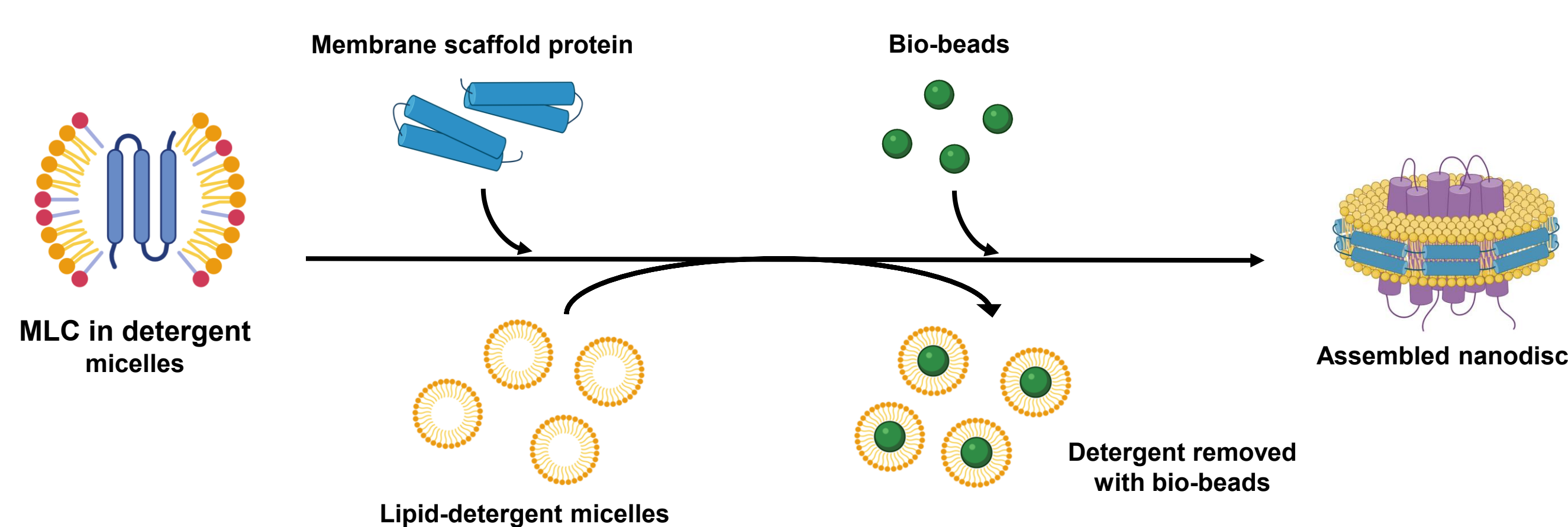


II. Buffer and detergent screening

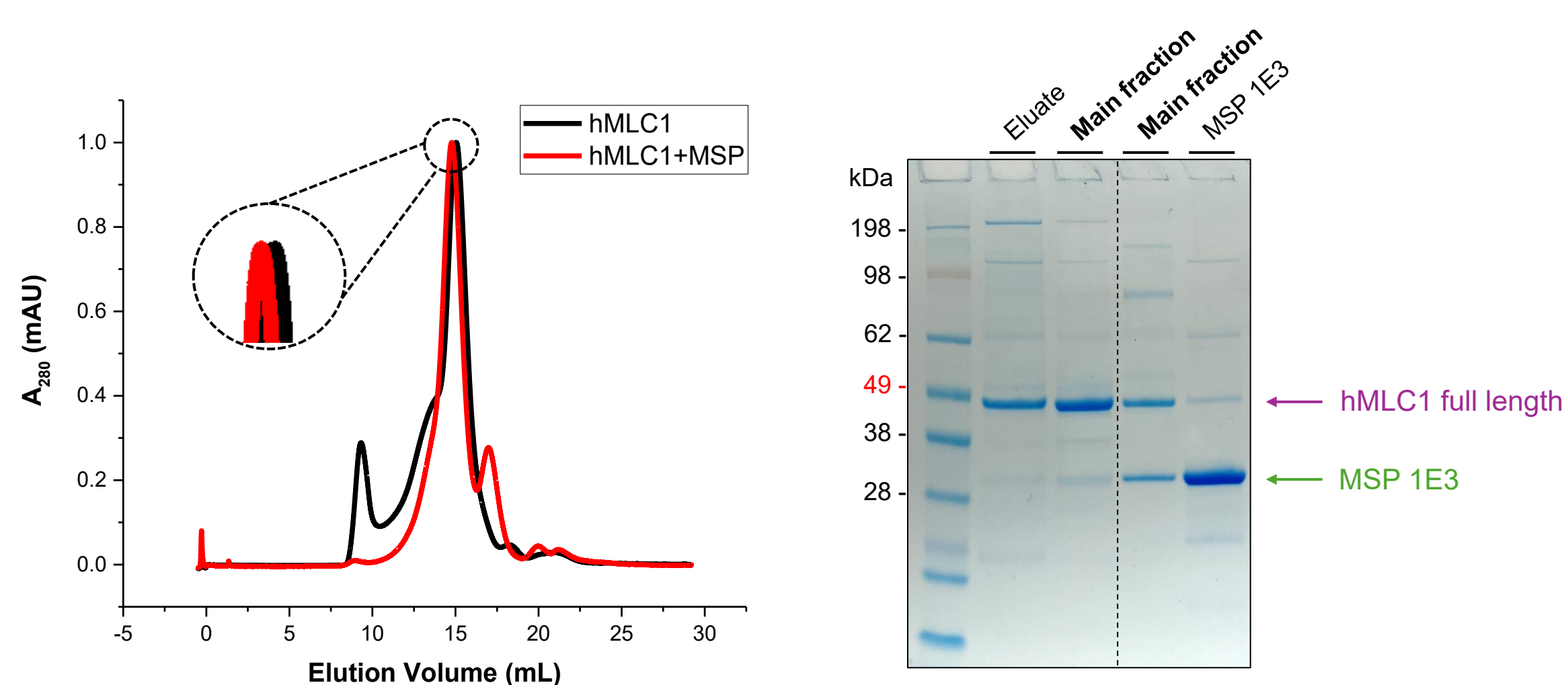


To optimize purification conditions for the MLC1 protein, we evaluated two factors: (I) expression in HEK293S GnTI⁻ cells to ensure homogeneous glycosylation and sodium butyrate treatment during transfection to enhance expression via transcriptional activation. (II) Buffer and detergent screening to improve purification efficiency and protein stability.

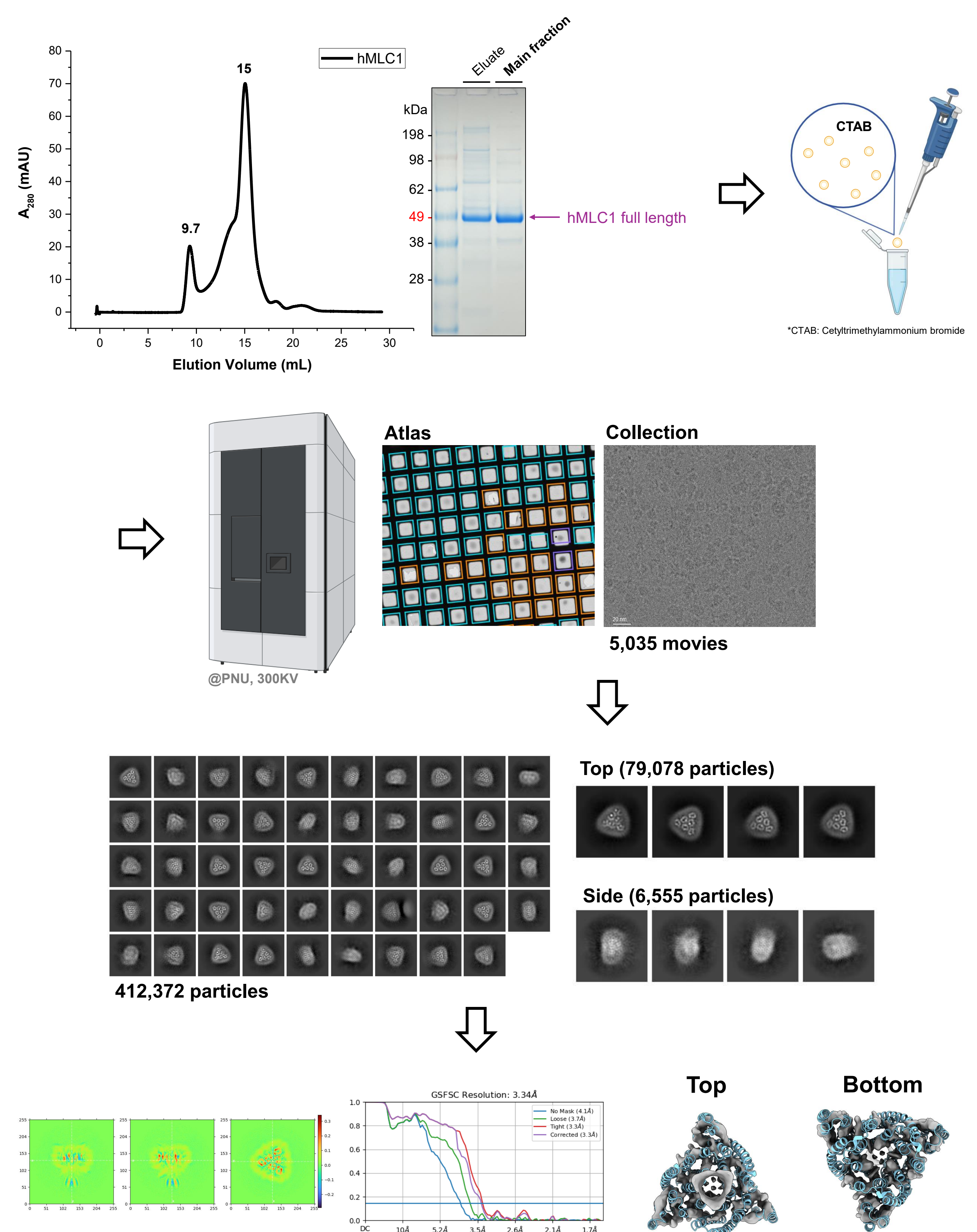
Reconstitution of MLC1 into nanodisc



For nanodisc reconstitution, purified MLC1 protein was combined with MSP1E3 and a phospholipid mixture at a molar ratio (protein:lipid:MSP). The mixture was incubated to allow the formation of nanodiscs, and detergent removal was carried out using Bio-Beads. This method enabled the successful incorporation of MLC1 protein into lipid bilayer nanodisc, providing a membrane-mimicking environment suitable for structural analysis.



Purification and cryo-EM structural study of MLC1



Summary and on-going research

To investigate the structural characteristics of the MLC1 protein, we expressed the protein in a mammalian cell line and systematically optimized the purification conditions. However, cryo-EM imaging of purified MLC1 showed preferred orientation, which limited high resolution structural determination. To overcome preferred orientation issues, we have tested additive treatment (varying concentration of CTAB) and nanodisc reconstitution. These approaches showed a possibility of sample a wider variety of particle orientations, but it was not enough to determine the atomic resolution structure of the MLC1 protein. We are currently optimizing conditions for obtaining additional views, particularly the side view of the protein, to achieve a more complete and detailed understanding of its structural architecture.

The Basic Science Research Program (NRF, 2021R1A2C1004884) and the KBRI basic research program (25-BR-01-02).