Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supplementary Information

Sortase mediated labelling of lipid nanodiscs for cellular tracing

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Methods

Materials

The lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids. Plasmid for expression of MSP1D1 (Plasmid #20061) was obtained from Addgene. Sortase A and TEV protease were purified as reported previously.[1]

Membrane Scaffold Protein Expression and Purification

Detailed MSP1D1 (MSP) expression and purification methods have been previously reported by the Sligar lab.[2] The cell preparation was conducted under sterile conditions. 2.0 µL of plasmid pMSP1D1 was added to 50 µL of competent *E. coli* BL21 Gold (DE3), incubated on ice for 30 min and heat-shocked at 42 °C for 45 s before returning on ice for a further 5 min. 200 µL of Lysogeny Broth (LB) media was inoculated with the cell/plasmid mixture and incubated at 37 °C, with shaking at 200 rpm, for 1 h. 200 µL of transformed cells were plated on Agar plates containing kanamycin (50 µg/mL) and incubated overnight at 37 °C, with shaking at 200 rpm.

A single colony of *E. coli* BL21 Gold (DE3) cells harbouring the pMSP1D1was used to inoculate LB media (10 ml, 50 µg/mL kanamycin). The starter culture was incubated overnight at 37 °C before adding it to Terrific Broth (TB) or Auto Induction Media (AIM) media (1 L, 50 µg/mL kanamycin). For TB media – the culture was grown at 37 °C and induced with 1 mM IPTG (isopropylthio-β-galactoside) when the optical density (OD600) reached 2.5. One hour after induction, the temperature was lowered to 28 °C. Four hours after induction, the cells were harvested. For AIM media - the culture was grown at 20 °C for 24 h before harvesting. The cells were isolated by centrifugation (10000 rpm, 10 min), the supernatant discarded and the bacterial pellet stored at -80 °C.

The frozen cell pellet was resuspended in lysis buffer (50 mL; 20 mM phosphate buffered saline (PBS) pH 7.4; after resuspending the cells, 10% Triton X-100 was added to make 1%) and left on ice for 30 min. The suspension was mechanically disrupted using a Constant Systems Cell disruptor (35 kpsi, 4 °C), the lysate was cleared by centrifugation (48000×kg, 20 min), the pellet discarded and the supernatant ran on a Ni-NTA column (Qiagen, at 4 °C) pre-equilibrated with PBS buffer. The column was washed sequentially with three sequential Tris buffered solutions (3× column volume (CV) each): i) 40 mM Tris, 0.3 M NaCl, 1% Triton X-100, pH 8.0; ii) 40 mM Tris, 0.3 M NaCl, 50 mM Na-Cholate, 20 mM Imidazole, pH 8; iii) 40 mM Tris, 0.3 M NaCl, 50 mM Imidazole, pH 8.0. The protein was eluted with elution buffer (40 mM Tris, 0.3 M NaCl, 0.4 M Imidazole, 40 mM Na-Cholate, pH 8.0; 1×CV). Protein-containing fractions and their purity were identified by SDS-PAGE. The protein concentration was measured by UV spectroscopy at 280 nm, using a Thermo Scientific NanoDrop 2000 and a theoretical extinction coefficient of 21430 M^{-1} cm^{-1}.

Nanodisc Assembly and Characterisation Protocols

We used a slightly modified nanodisc assembly protocol compared to that reported by the Sligar lab:[2] we add the MSP to the lipid during the lipid resuspension step rather than following the formation of lipid-detergent micelles.

The concentration of stock POPC in chloroform solutions were measured by a total phosphorous assay. An appropriate volume of lipid dissolved in chloroform was dried to a thin film under vacuum. The prepared lipid was then incubated with a solution of MSP stabilised in Na cholate micelles (MSP in elution buffer) (1:65:100 MSP to lipid to Na cholate molar ratio), by gentle shaking for 2.5 h at 4 °C, close to the transition temperature of the lipid. An appropriate amount of wet bio-beads (0.3 g per mL of nanodiscs), previously washed with 2 volumes of methanol and 4 volumes of buffer (40 mM Tris pH 8, 0.3 M NaCl), were added. The mixture was incubated for 5-6 h at 4 °C. The bio-beads were then separated by careful pipetting.

Nanodisc size distributions were analysed by size-exclusion chromatography (SEC) experiments using a Superdex 200 10/300 chromatographic separation column and run using an ÄKTApurifier™ system (GE Healthcare). Dynamic light scattering (DLS) was also employed as a complementary technique for size analysis using a Zetasizer Nano (Malvern) particle size analyser with Zetasizer software.

Sortase A labelling method

Sortase A (SrtA) (480 μM) and fluorescein depsipeptide (2 mM) were prepared in HEPES buffer (50 mM HEPES, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.5). Prior to the SrtA mediated ligation, TEV protease was used for the cleavage of the His tag attached to the N-terminus of the MSP. Nanodiscs (137 μL, 27 μM[*]) were incubated overnight in the presence of TEV protease (2.74 μL, 1.0 μM final concentration), at 4 °C. The reaction was monitored by mass spectrometry (MS), however complete cleavage was not observed. The nanodiscs were separated from the protease by SEC.

SrtA (8.6 μM final concentration, 480 μM stock concentration), nanodiscs (27 μM) and fluorescein depsipeptide (5 equivalents, 2 mM stock concentration) were mixed together to form a total volume of 55.5 μL. The mixture was then left at room temperature. After 6 h, an additional 4 equivalents of depsipeptide were added and the incubation continued overnight. The reaction mixture was monitored by MS and SDS-PAGE. SDS-PAGE gels were imaged using EPI-white illumination in the BIO RAD Molecular Imager® Gel Doc™; gels containing fluorescently labelled MSP1D1 were imaged by UV illumination prior to staining. Once the MSP had been modified, the nanodisc mixture was immediately purified using Nickel resin. The reaction mixture was incubated with Nickel affinity resin (Qiagen) for up to 4 h, by gently shaking. The mixture was subsequently separated from the resin by centrifugation and careful pipetting, and washed with buffer (40 mM Tris pH 8, 0.3 M NaCl).

Mass Spectrometry

Mass spectrometry analysis of nanodiscs without His-tag and fluorescein-nanodiscs was done using a Bruker MicrOTOF accurate MS system. Samples were diluted to 50% (v/v) with water before being loaded directly into the instrument. Prior to mass spectroscopy analysis, the samples were automatically diluted with 0.1% TFA, 50% MeCN (v/v) in water. Processing and deconvolution of multiple charge states was performed using BioPharma Compass 1.1 Software.

[*] Nanodisc concentration is calculated as half the MSP concentration. This is due to nanodisc structure, which contains two copies of the MSP protein.

Imaging of Intracellular Delivery

HeLa cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with foetal bovine serum (10% *v/v*) and PenStrep (10000 U mL^{-1} penicillium and 10 mg mL^{-1} streptomycin; 1% *v/v*). Once confluent, cells were released from the surface of the flask by treatment with 2.0 mL 0.25% (*w/v*) trypsin in PBS at 37 °C for 5 min, and counted using a haemocytometer. Cells were plated at 1.5 × 10^5 cells/well on round coverslips in 12-well plates in 1.0 mL (per well) supplemented DMEM. At approximately 70% confluency, the medium was replaced with fresh supplemented DMEM and the cells were incubated with 2.0 µL fluorescein-labelled nanodisc samples (25 nM final nanodisc concentration when added to the cells) or left untreated. After 4 h and 24 h, the medium was removed and cells were washed with 2 × 1.0 mL PBS, fixed with 4% (*v/v*) paraformaldehyde in PBS for 10 minutes and washed again with 2 × 1.0 mL PBS. The cells were mounted onto a glass slide with Prolong® Gold Antifade Reagent with DAPI (15 µL) for nuclei staining. The slides were set overnight in the dark before being sealed with nail varnish to preserve the cells. Confocal microscopy images were acquired on a Zeiss LSM510 META upright confocal microscope.

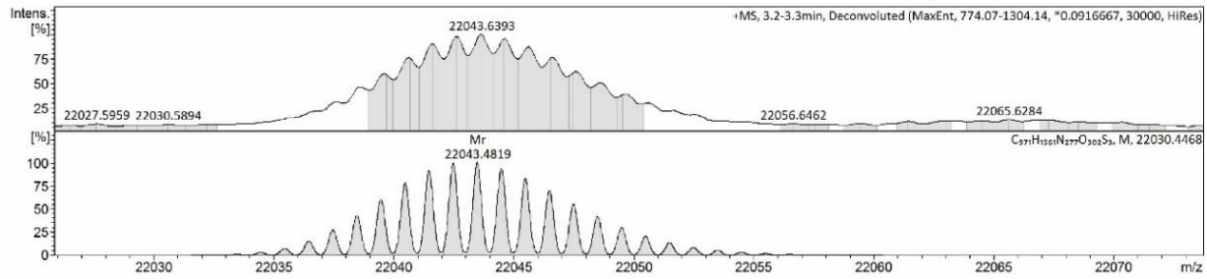

Figure S1. Mass spectrum of the MSP1D1 within nanodiscs, after His-tag cleavage (upper image), and simulation of the MSP1D1 without the His-tag and TEV protease recognition sequence, based on the chemical formula calculated from the amino acid sequence of MSP1D1 (bottom image).

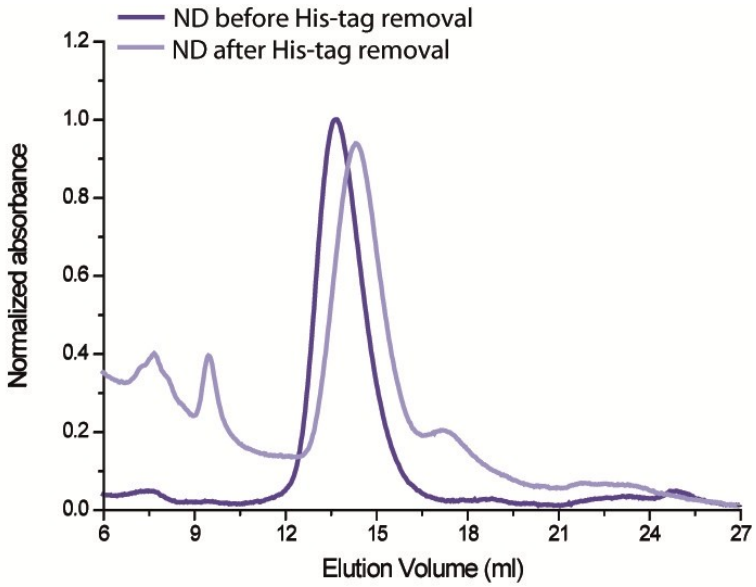

Figure S2. Size exclusion chromatogram showing that nanodiscs with cleaved His-tags could be resolved from uncleaved nanodiscs, where the lower total molecular weight of the His tag cleaved nanodiscs leads to a greater elution volume (and longer retention time).

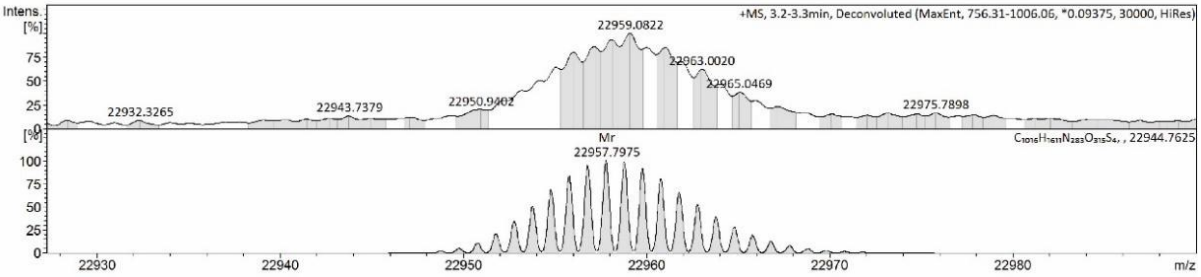

Figure S3. Mass spectrum of the fluorescein-modified MSP1D1 within nanodiscs (upper image), and simulation of the modified MSPD1, based on the the amino acid sequence of MSP1D1 and the fluorescein-depsipeptide (bottom image).

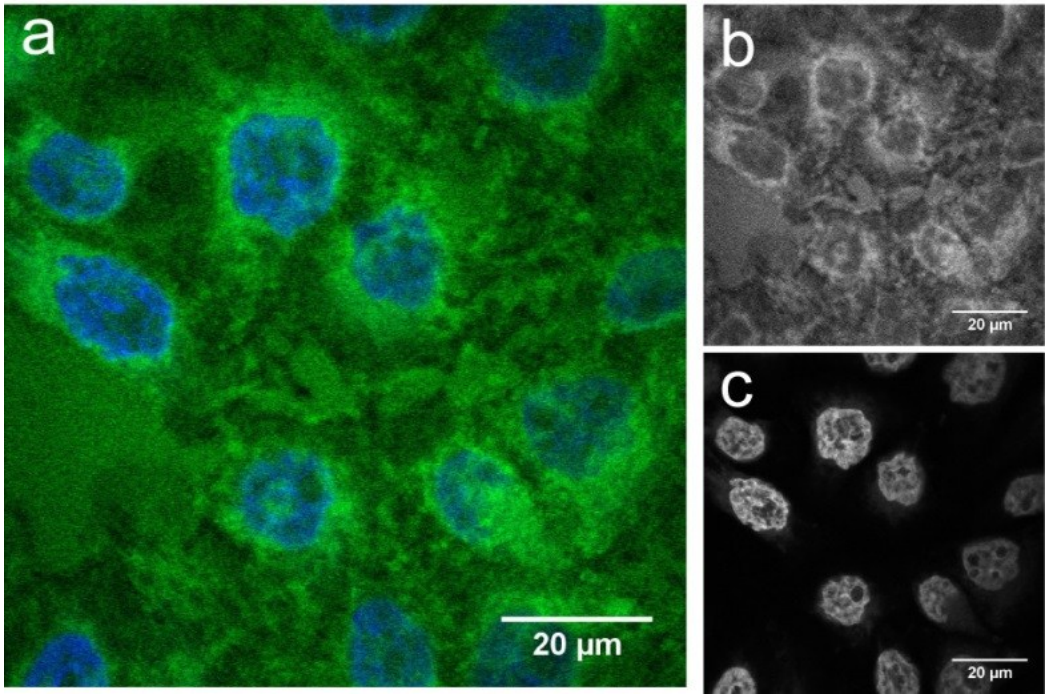

Figure S4. As a control, HeLa cells were incubated with unlabelled nanodisc assemblies for 4 h at 37 °C. The cell nuclei were labelled with DAPI (blue). a. Merged image; b. FITC channel showing background cellular autofluorescence and no punctate spots corresponding to the nanodiscs; c. DAPI

1. D. J. Williamson, M. A. Fascione, M. E. Webb and W. B. Turnbull, *Angewandte Chemie-International Edition*, 2012, **51**, 9377-9380.

2. T. K. Ritchie, Y. V. Grinkova, T. H. Bayburt, I. G. Denisov, J. K. Zolnerciks, W. M. Atkins and S. G. Sligar, in *Methods in Enzymology; Liposomes, Pt F*, ed. N. Duzgunes, 2009, vol. 464, pp. 211-231.