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Sortase-mediated labelling of lipid nanodiscs for cellular tracing†

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Lipid nanodiscs have broad applications in membrane protein assays, biotechnology and materials science. Chemical modification of the nanodiscs to expand their functional attributes is generally desirable for all of these uses. We present a method for site-selective labelling of the N-terminus of the nanodisc's membrane scaffold protein (MSP) using the Sortase A protein. Labelling of the MSP was achieved when assembled within the lipid nanodisc architecture, demonstrating that this method can be used as a retrofit approach to modification of preformed nanodiscs before or during application. We label the MSP with a fluorescent fluorescein moiety and use them to image nanodisc uptake into HeLa cells. The Sortase A labelling method could be employed as a general approach to labelling nanodiscs with application-specific functionalities.

Lipoprotein and lipoprotein-mimetic particles have recently attracted significant interest in the field of biotechnology.^{1,2} This is due to their potential for applications in areas such as the stabilisation and handling of membrane proteins³ and formulation of active pharmaceutical ingredients in nanomedical delivery technologies.^{4–11} These nanoscale particles, which we will refer to as lipid nanodiscs, consist of a discotic segment of lipid bilayer membrane where the hydrophobic line tension at the disc perimeter is stabilised by an amphipathic protein or polymer, e.g. natural apolipoproteins,¹² synthetic apolipoprotein derivatives,^{13,14} or styrene maleic acid copolymers.^{15,16}

Chemical functionalisation of lipid nanodiscs for biotechnology applications is highly desirable as this facilitates the incorporation of new, additional functionalities within these particles. Examples include the addition of image contrast agents,¹⁷ targeting ligands,¹⁸ or environmental sensors. These functionalities could be attached to the lipids in the bilayer, for example DNA-functionalised lipids have been used to adapt nanodiscs for directed assembly into

quasi-1D columnar stacks, which were used as templates to direct gold nanoparticle assembly.^{19,20} Similarly, the scaffold proteins (or polymers) are desirable substrates for functionalisation. The scaffold proteins of lipid nanodiscs have previously been functionalised with tetramethylrhodamine dyes by engineering a cysteine into the protein for site-specific labelling.¹⁷ However, labelling strategies for these proteins that can be performed on the original construct, without the need to reengineer and express a batch of new protein, would be highly attractive.

Here we present a method for specifically labelling the N-terminus of the MSP1D1 scaffold proteins of lipid nanodiscs. MSP1D1 (MSP hereafter) is a truncated derivative of apolipoprotein A-1, developed in the laboratory of Stephen Sligar.²¹ To achieve site specific labelling, we use the Sortase A (SrtA)-mediated labelling and the optimised depsipeptide substrates previously reported by Williamson *et al.*²² SrtA is a transpeptidase required for the attachment of proteins to the cell wall of Gram-positive bacteria but can also be used for linkage of peptides and proteins using C-terminal or internal LPXTG motifs in one substrate together with an N-terminal glycine in the second substrate. Replacement of the LPXTG peptide sequence with LPXTOG depsipeptide leaves the labelling reaction irreversible and can be used for quantitative labelling.²³ For SrtA-mediated labelling to be effective, the target protein must possess an N-terminal glycine residue. The expressed MSP scaffold protein has a His-tag, used for affinity purification, linked to the scaffold protein by a Tobacco Etch Virus (TEV) protease recognition site (ENLYFQG). We therefore used the TEV protease to cleave the His-tag from the scaffold protein, which hydrolyses the amide bond between the QG amino acid residues. This conveniently results in a glycine residue at the N-terminus of MSP, as required.

As a proof-of-principle for SrtA-mediated labelling of lipid nanodiscs, we functionalised MSP with a fluorescent fluorescein-depsipeptide. The MSP1D1 scaffold protein was overexpressed in *E. coli* BL21 (DE3) from the expression plasmid reported by Sligar and coworkers²⁴ and purified by sequential Ni-NTA affinity chromatography and size-exclusion chromatography. Our initial attempts to directly label monomeric MSP using SrtA were

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unsuccessful due to protein precipitation during both the TEV protease cleavage and SrtA labelling steps. We therefore chose to directly label MSP in pre-formed lipid nanodiscs.

Lipid nanodiscs were formed using a slightly modified method compared to previously reported protocols (Fig. 1a).²⁴ Previous protocols have used two-step formation of lipid-detergent micelles followed by addition of the scaffold proteins. In our optimised protocol, the detergent-solubilised MSP solution was added directed to the dried lipid (POPC) film in a MSP:lipid:cholate ratio of 1:65:100 and incubated with gentle mixing at 4 °C for 2.5 h. The sodium cholate detergent is then removed by incubation with 0.3 g ml⁻¹ of wet Bio-beads at 4 °C for 4 to 5 h. We found that storage of MSP in detergent-containing buffer (40 mM sodium cholate) improved the stability of the protein against aggregation, which can hinder nanodisc formation once protein aggregates in stock solutions become large.

The expected size distribution of MSP lipid nanodiscs was confirmed by size exclusion chromatography (SEC) and dynamic

light scattering (DLS) (Fig. 1b and c). Nanodiscs eluted at a similar volume to a 150 kDa alcohol dehydrogenase molecular weight marker in the SEC with a narrow size distribution, as has previously been reported for MSP nanodiscs.²¹ The average hydrodynamic diameter of nanodiscs was measured to be 10.1 nm by DLS, close to the 9.7 nm diameter previously reported by SAXS measurements.²¹ Furthermore, we analysed the MSP stock solution by DLS, which showed a bimodal size distribution with peaks at 1.7 nm and 6.5 nm hydrodynamic diameters. This is consistent with the presence of monomeric and small oligomeric protein populations; the presence of the cholate detergent inhibited the formation of larger MSP aggregates, which inhibit efficient nanodisc formation.

Two approaches were taken to fluorescence labelling of the nanodiscs; either labelling MSP before or after nanodisc formation. In practice, labelling MSP prior to nanodisc formation was impractical due to the prolonged incubation times required for both TEV protease and sortase-mediated labelling as detailed below. The final SrtA-mediated nanodisc labelling protocol is schematically outlined in Fig. 2a. Following nanodisc formation, the His-tag of MSP was cleaved with TEV protease by overnight incubation at room temperature. Complete cleavage was confirmed by mass spectrometry following disassembly of the nanodiscs (Fig. S1, ESI†). The size change of the nanodiscs following His-tag cleavage was also resolvable by SEC: nanodiscs with cleaved His-tags elute slightly later from the column than nanodiscs with the His-tags intact (Fig. S2, ESI†). The cleaved MSP



Fig. 1 Lipid nanodisc formation and characterisation. (a) Schematic diagram of our nanodisc formation protocol from MSP and lipids coated on glass. (b) Size exclusion chromatogram of lipid nanodiscs and an alcohol dehydrogenase molecular weight marker. (c) Hydrodynamic size distributions of lipid nanodiscs and the MSP1D1 scaffold protein obtained by DLS show that assembled nanodiscs are monodisperse.

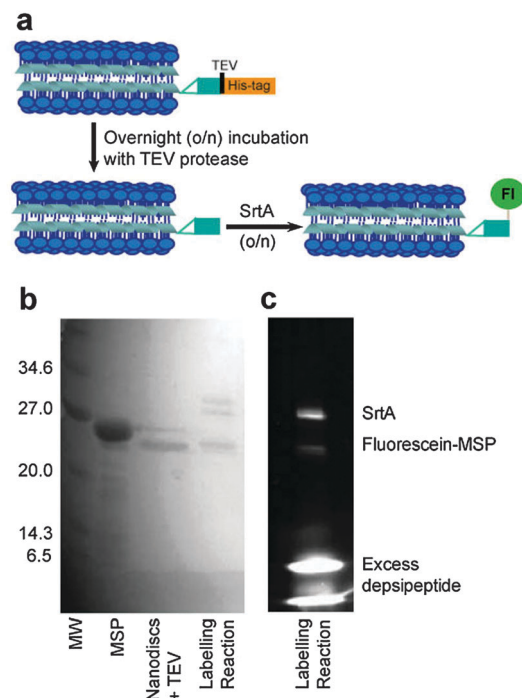


Fig. 2 Sortase A-mediated labelling of lipid nanodiscs. (a) Schematic representation of the labelling protocol. Fl = fluorescein-label. (b) SDS-PAGE gel of MSP1D1 and modified derivatives: lane 1: MSP1D1; lane 2: MSP1D1 in nanodiscs with the His-tag removed; lane 3: fluorescein-MSP1D1 nanodiscs. (c) Fluorescent image of the SDS-PAGE gel lane 3 showing successful labelling of the MSP.



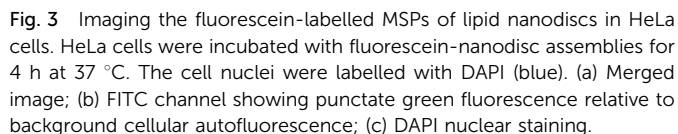


Fig. 3 Imaging the fluorescein-labelled MSPs of lipid nanodiscs in HeLa cells. HeLa cells were incubated with fluorescein-nanodisc assemblies for 4 h at 37 °C. The cell nuclei were labelled with DAPI (blue). (a) Merged image; (b) FITC channel showing punctate green fluorescence relative to background cellular autofluorescence; (c) DAPI nuclear staining.

To demonstrate the potential application of fluorescein-functionalised nanodiscs, we investigated their traceable delivery into mammalian cells. Labelled nanodiscs were incubated with HeLa cells for 4 h before paraformaldehyde (4%) fixation, washing and imaging by confocal microscopy (Fig. 3). Bright, punctate spots of fluorescence inside these cells are clearly visible above the background autofluorescence in the green (fluorescein) channel that are not apparent in control images of these cell lines with the addition of unlabeled nanodiscs (Fig. S4, ESI[†]). These spots mark the location of the MSP scaffold protein, but not necessarily the location of the nanodisc lipids. Previous work by Carney *et al.* showing the delivery of nanodiscs containing Gd-labeled lipids to HeLa cells indicates that these lipids are dispersed throughout the cell membranes (implying dynamic exchange of lipids between the nanodiscs and the HeLa cell), while the MSP is localised as punctate spots inside the cell, in agreement with our observations.¹⁷ The brightness of these spots in

Our data demonstrates the uptake of lipid nanodiscs by HeLa cells, showing their potential as carriers for intracellular delivery. These constructs will allow future detailed mechanistic studies of nanodisc uptake in different cell lines using organelle-specific immunostaining. In particular it will allow investigation of how the scaffold proteins are specifically trafficked by these cells.

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