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In Vitro Glycosylation of the Membrane Protein γ-Sarcoglycan in Nanodiscs

Michael S. Harris, Rachel F. Dolan, James R. Bryce, Jonas G. Ewusi, and Gabriel A. Cook*

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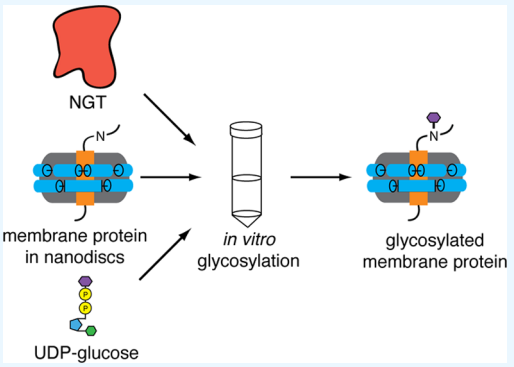
ABSTRACT: Membrane glycoproteins are proteins that reside in the membranes of cells and are post-translationally modified to have sugars attached to their amino acid side chains. Studies of this subset of proteins in their native states are becoming more important since they have been linked to numerous human diseases. However, these proteins are difficult to study due to their hydrophobic nature and their propensity to aggregate. Using membrane mimetics allows us to solubilize these proteins, which, in turn, allows us to perform glycosylation *in vitro* to study the effects of the modification on protein structure, dynamics, and interactions. Here, the membrane glycoprotein γ-sarcoglycan was incorporated into nanodiscs composed of long-chain lipids and membrane scaffold proteins to perform N-linked glycosylation in which an enzyme attaches a sugar to the asparagine side chain within the glycosylation site. We previously performed glycosylation of membrane proteins *in vitro* when the protein had been solubilized using different detergents and short-chain lipids. This work demonstrates successful glycosylation of a full-length membrane protein in nanodiscs providing a more biologically relevant sample to study the effects of the modification.



INTRODUCTION

Protein glycosylation, the attachment of sugars to their amino acid side chains, is the most common post-translational modification (PTM) and allows our cells to diversify the limited number of proteins they contain.[1−4] While this diversity is imperative for the array of functions of these proteins, it creates a complicated problem for scientists as we attempt to use methods that require homogeneous samples to elucidate the effects of these modifications. In addition to the heterogeneity problem, some methods require milligram amounts of protein (i.e., NMR, crystallography, etc.) which are most commonly expressed through recombinant bacterial approaches or cell-free systems, neither of which produce protein that is glycosylated. Previously, several groups had shown that recombinantly expressed and purified soluble proteins could be glycosylated *in vitro* using recombinantly expressed *N*-glycosyltransferases.[5−10] Using similar methods, our group has previously shown that it is also possible to glycosylate membrane proteins in detergent and lipid micelle environments using an N-glycosyltransferase from *Actinobacillus pleuropneumoniae* (ApNGT).[10] The enzyme recognizes the consensus sequence N-(X)-T in which X is any amino acid besides proline and attaches the sugar to the side chain of the asparagine residue.

Membrane proteins, found in the membranes of cells, are an important set of proteins. They often work as transporters, ion channels, and signaling proteins, and are members of large multiprotein complexes.[11] They also play significant roles in many diseases such as cystic fibrosis, Parkinson's, Alzheimer's, and atherosclerosis.[12] One such protein, γ-sarcoglycan, chosen for this work, is important for maintaining the integrity of the muscle cell sarcolemma and is linked to muscular dystrophy.[13,14] The ability to study its different glycosylation states will be vital in gleaning information about this protein and how it plays a role in muscular dystrophy. Due to membrane proteins' propensity to aggregate in aqueous solutions, membrane proteins have been difficult to study, and knowledge about this subset of proteins is lacking compared to their soluble counterparts. Sample preparation of hydrophobic membrane proteins has evolved over the last 40 years through the use of micelles, proteoliposomes, bicelles, and, more recently, lipid nanodiscs.[15−17]

Nanodiscs, a combination of long-chain lipid and amphipathic membrane scaffolding protein (MSP), self-assemble into bilayer discs that provide an environment for the integral membrane proteins to reside.[18,19] There are several benefits of using nanodiscs for these studies. These samples are devoid of free detergents and lipids that could inhibit the

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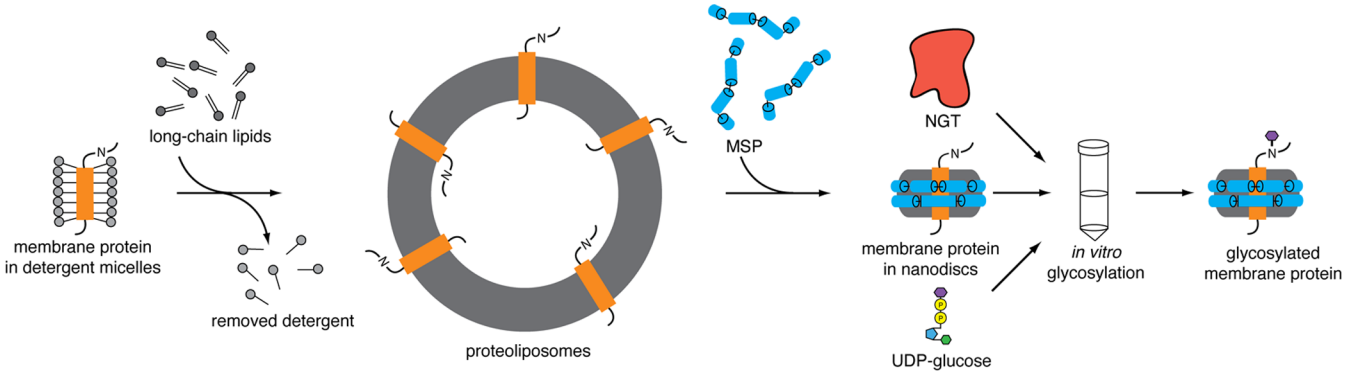
Scheme 1. Schematic of Membrane Protein/Nanodisc Formation and Glycosylation[a]



[a]Long-chain lipid is added to purified membrane protein in detergent micelles. The detergent is removed by dialysis to form proteoliposomes. Once the detergent is removed MSP is added to the proteoliposomes resulting in the spontaneous formation of protein-containing nanodiscs. For N-linked glycosylation, NGT and UDP-glucose are added to the protein/nanodisc sample. Successful glycosylation will result in a sugar being attached to the membrane protein.

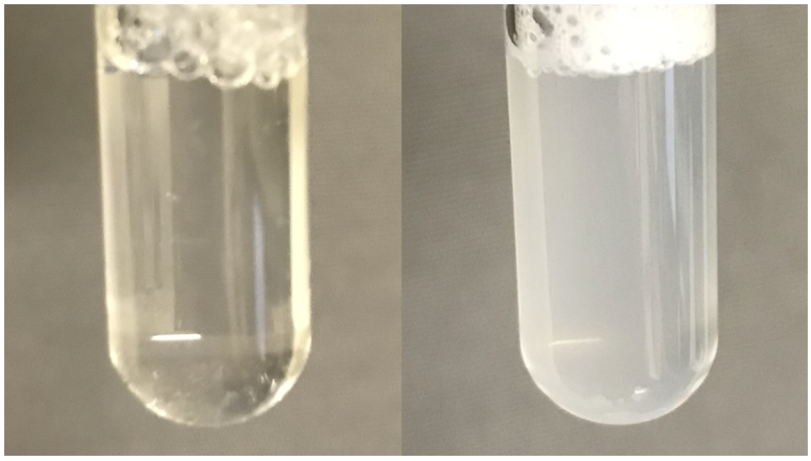


Figure 1. Formation of γ-sarcoglycan/DMPC proteoliposomes. Left, the sample before dialysis used to remove the DDM detergent from the protein/detergent/lipid mixture. Right, the same sample postdialysis displaying the pearlescent appearance indicating that the detergent had been successfully removed and the formation of proteoliposomes had occurred.

activity of the glycosylating enzyme, since all of the detergent is removed during the incorporation of the protein into the proteoliposomes and all of the lipids that remain are those that are incorporated into the nanodiscs. For many proteins, the composition of the membrane that it resides in within the cell is important for its structure and function.[20−22] The headgroups, chain lengths, saturation, and composition of the chains of the lipids can influence the protein. The lipids used in the discs determine the thickness of the bilayer. Using nanodiscs allows for the use of a large variety of lipids and in some cases sterols to better mimic the membrane environment. The diameter of the nanodiscs can also be adjusted by varying the length of the scaffolding protein that is used, with the typical size ranging from 10 to 30 nm.[23,24] The shape and dimensions of the nanodiscs can accommodate supramolecular complexes.[25−27] Interestingly, the structure of the oligosaccharyltransferase (OST) complex was determined in nanodiscs.[28] For glycosylation studies in particular, protein complexes where one or more of the glycoproteins are glycosylated and how this modification affects the way these proteins interact and function can be studied. Another benefit of working with nanodiscs is that they do not preferentially orient. This allows for transmembrane proteins to be accessible by the glycosylating enzymes on both sides of the bilayer. This is a problem that other lipid assemblies, particularly liposomes, cannot circumvent.

Protein-nanodisc samples have been prepared in several ways. The two most common are the following: (1) adding MSP to microsomal membrane preparations[29] and (2) adding MSP to proteoliposomes that were prepared from purified protein samples.[23,30] In this work, since the protein γ-sarcoglycan was purified from inclusion bodies, proteoliposomes were prepared, and these were converted into nanodiscs by the addition of pure MSP. It has been established that the methods are protein dependent, and efforts should be made to establish which method works best for a particular protein.

RESULTS AND DISCUSSION

The purpose of this study was to determine the feasibility of glycosylation of membrane proteins in membrane mimetic nanodiscs through N-linked *in vitro* glycosylation using the methods illustrated in Scheme 1. For this study, we used the membrane protein γ-sarcoglycan and the N-glycosylating enzyme ApNGT. The two proteins were separately expressed in *Escherichia coli* and purified for these experiments. Purified γ-sarcoglycan was used as it can be studied as a simple model.

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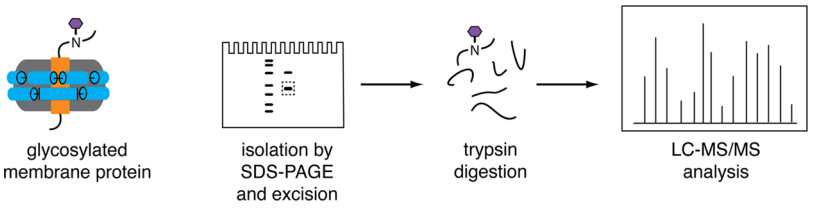
Scheme 2. Schematic of Glycosylation Analysis of a Membrane Protein in Nanodiscs[a]



[a]Glycosylation samples are run on an SDS-PAGE gel so that the glycosylated protein can be isolated. The gel band representing the glycoprotein is subjected to trypsin digestion and run on LC-MS/MS to determine if glycosylation was successful, evidenced by an increase in mass of the glycosylated peptide.

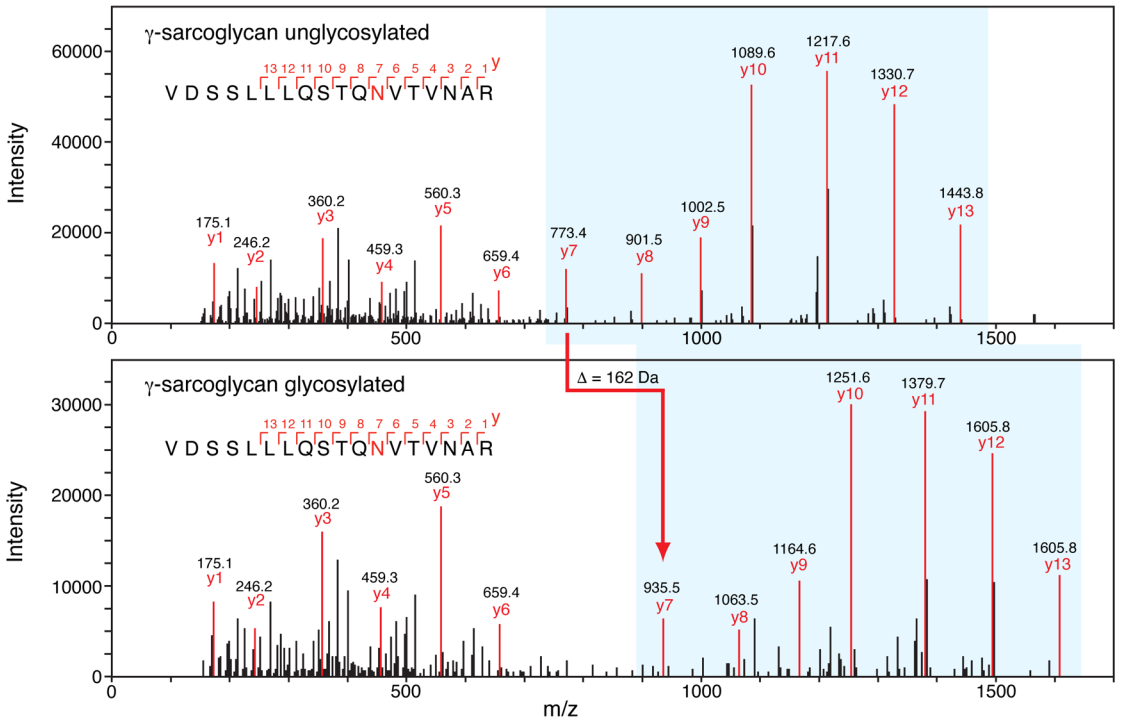


Figure 2. Confirmation of *in vitro* glycosylation of γ-sarcoglycan by LC-MS/MS. Using LC-MS/MS, the peptide VDSSLLLQSTQN(Glc)-VTVNAR was isolated and fragmented in both samples. Top, the unglycosylated control sample. Bottom, the glycosylated sample highlighting the increased mass of peptide fragments y7 through y13. The increase of approximately 162 Da indicates that the sugar was attached at the glycosylation site. The blue shading highlights the fragments of the peptide that have a larger mass in the glycosylated sample.

This is because it has a single transmembrane domain and single N-linked glycosylation site. This protein was added to nanodiscs using a proteoliposome-to-nanodisc approach. The protein was first successfully incorporated into DMPC proteoliposomes using the methods described previously for the G-protein-coupled receptor CXCR1, by Park et al.[31] Purified protein was dissolved in detergent and added to a dried film of lipid. The mixture was subjected to dialysis to slowly remove the detergent and to force the formation of protein-containing proteoliposomes as described by Strickland et al.[32] The removal of detergents was determined by the presence of a translucent solution (Figure 1) since liposome samples, devoid of detergent, tend to form translucent suspensions.[33] These suspensions have yielded translucent pellets and clear supernatants under high-speed centrifugation as a confirmation of liposome formation. The proteoliposome sample was then removed from dialysis, and purified MSP was added to the sample. The addition of the scaffolding protein caused the solution to transition to a completely clear solution, indicating that the nanodiscs had formed and that the protein containing the liposomes had been fully incorporated.

Once incorporated into nanodiscs, the protein was subjected to *in vitro* glycosylation using ApNGT and UDP-glucose as the sugar donor. The reaction mixtures were left at room temperature overnight to allow for the attachment of glucose to the single asparagine residue. The reaction sample and the unglycosylated control sample were analyzed by mass spectrometry to determine if *in vitro* glycosylation was successful as illustrated in Scheme 2. The glycosylated and control samples were run on separate SDS-PAGE gels to isolate the γ-sarcoglycan protein from other components in the mixtures so that they could be subjected to digestion by trypsin. This trypsinization method with SDS-PAGE gels was a vital step in the analysis of the transmembrane protein. Like many other membrane proteins, it is not readily soluble in common mass spectrometry solvents due to its hydrophobic nature. Once the γ-sarcoglycan band was excised from the gel, the protein underwent trypsin digestion and was then analyzed by LC-MS/MS to isolate peptide sequences that corresponded

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to the glycosylation site. It was determined that the peptide corresponding to residues 98−115, VDSSLLLQSTQNVTVNAR, could be used for determining whether the γ-sarcoglycan protein was glycosylated. This peptide contains the only N-linked glycosylation site within γ-sarcoglycan. The sugar attachment occurs at the asparagine (N109) that is followed by the amino acids valine (V110) and threonine (T111).

As shown by the spectra in Figure 2, peptide spectral matches in the control sample and the reaction mixture showed that fragments representing the region downstream of the glycosylation site (y1-y6) all had the same mass in both samples. However, in the reaction mixture, the amino acids upstream from the region that included the glycosylation site (y7-y13) had masses that were 162 Da higher than the same sequences in the control sample, indicating the presence of the glycosylated peptide VDSSLLLQSTQN(Glc)VTVNAR. For example, peptide y7 increased in mass from 773.4 Da in the control sample to 935.5 Da in the glycosylated sample, indicating that the asparagine residue within the glycosylation site had been modified by the enzyme.

CONCLUSIONS

The use of lipid nanodiscs has been widely accepted as one of the most accurate membrane mimetics to study the native conformations of membrane proteins. These protein−lipid assemblies could yield samples that will allow us and other scientists to accurately measure the structure, dynamics, and interactions of proteins that have been post-translationally modified. We can also use these samples to identify changes in these proteins that result from the addition of the PTM substituent. Such studies will benefit from the homogeneous nature of these systems and allow us to directly compare glycosylated and unglycosylated samples by using NMR and other spectroscopic methods.

After demonstrating the first successful *in vitro* glycosylation of a full-length membrane protein in lipid nanodiscs here, we will attempt to use the same methods for other membrane glycoproteins involved in human diseases. Of course, this is only the first example that the methods are feasible, and optimization of the nanodisc composition and the enzyme activity will be necessary for maximizing *in vitro* glycosylation efficiency in future samples.

This method of N-linked glycosylation of membrane proteins may also act as a basis for future dynamic studies of glycosylated membrane proteins in nanodiscs. This initial methodology is currently being used for membrane proteins with a single glycosylation site, but eventually, as more studies are performed and more information is gleaned on the topic, it can be used on proteins and even protein complexes with multiple N-linked glycosylation sites. The possibilities with this capability are numerous and can lead the way as a building block in studies of membrane proteins and their different glycosylation states in a membrane mimetic that is widely accepted as one of the most accurate representations.

METHODS

Expression and Purification of MSP. The pET28-His-MSP1D1D5 plasmid for the expression of MSP was received as a gift from the laboratory of Stanley Opella (UCSD). This particular MSP expression vector was used since it expresses a scaffolding protein that contains both the N-terminal His_6-tag for purification and the TEV cleavage site for removal of the His_6-tag.[19,24] For expression of the MSP, 1 L of medium was prepared in a baffled culture flask with 1 L of deionized H_2O and 25 g of dry Luria−Bertani (LB) broth. Kanamycin was prepped by solubilizing 150 mg of dry kanamycin with 5 mL of deionized H_2O and was vortexed until dissolved. A 5 mL cell starter was created in a 50 mL conical centrifuge tube by combining 5 mL of LB broth, 5 μL of the sterile kanamycin, and 10 μL of MSP cell stock. The cell starter was placed on a shaker at 37 °C for 2 h. Fifty microliters of LB broth was taken from the 1 L flask and transferred to a 250 mL flask. Fifty microliters of kanamycin and 200 μL of the cell starter culture were added to 50 mL of LB broth. The 50 mL culture was placed on a shaker overnight (16 h) at 37 °C.

After 16 h, the 50 mL culture was removed from the shaker and was transferred to 1 L of LB broth. One milliliter of kanamycin was added to the flask, and it was placed on the shaker at 37 °C for approximately 4 h. When the optical density of the culture reached 0.600 at 600 nm, 120 mg of isopropyl β-D-1-thiogalactopyranoside (IPTG) in deionized H_2O was filter sterilized and added to the flask. After 4 h, the cell culture from the 1 L flask was transferred to a large centrifuge bottle and centrifuged at 8000*g* for 25 min at 4 °C. The cell pellet was collected and transferred to a 50 mL conical centrifuge tube and stored at −80 °C.

The cell pellet was defrosted on ice. Thirty milliliters of lysis buffer (20 mM phosphate buffer at pH 7.4) was added to the conical centrifuge tubes and vortexed until it was a homogeneous mixture. The cells were lysed by 4 min of sonication (2 s on, 8 s off). The lysate was transferred to a high-speed centrifuge tube, balanced, and centrifuged for 25 min at 35,000 ×g at 4 °C. The supernatant, containing unpurified MSP, was saved and prepared for column purification.

The MSP sample was subjected to gravity nickel affinity chromatography. Before the sample was run, the supernatant was filtered through a 0.20 μm syringe filter. The sample was run on 5 mL of Ni-NTA resin that was charged with 25 mL of 0.1 M $NiSO_4$. In addition, 25 mL of deionized H_2O was run through the column to rinse unbound $NiSO_4$. To equilibrate the column, 25 mL of 40 mM phosphate buffer (pH = 7.4) was put through before loading the filtered MSP sample. After the protein was applied to the column, a series of wash buffers were run through the column to remove unbound and nonspecifically bound proteins. These washes include 25 mL of Wash "A" (40 mM Tris/HCl 1% Triton X-100, 300 mM NaCl, pH = 8.0), 25 mL of Wash "B" (40 mM Tris/HCl, 50 mM Na-cholate, 300 mM NaCl, 20 mM imidazole, pH = 8.0), and 25 mL of Wash "C" (40 mM Tris/HCl, 300 mM NaCl, 50 mM imidazole, pH = 8.0). Following the wash steps, the purified protein was eluted with 10 mL of elution buffer (40 mM Tris/HCl, 300 mM NaCl, 0.4 M imidazole, pH = 8.0). The elutant was collected in a 50 mL conical centrifuge tube and stored at 4 °C until it was dialyzed.

The eluent MSP was transferred to 3.5 kDa dialysis tubing and dialyzed against 1 L of dialysis buffer (20 mM Tris/HCl, 100 mM NaCl, 0.5 mM EDTA, pH = 7.4) for 24 h. The dialysis was changed after 3 h. After dialysis, the MSP sample was stored at 4 °C. The concentration of the MSP sample after dialysis was determined using UV absorbance at 280 nm. The extinction coefficient of MSP at 280 nm is 21,430 M^{-1} cm^{-1}. The MSP sample was concentrated as needed to 4−12 mg/mL using a 10,000 MWCO centrifugal filter concentrator that was

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spun at 4000*g* at 4 °C. A stock solution of sodium azide was added to the sample to make a 0.01% (v/v) solution. The sample was stored at −20 °C.

For tobacco etch virus (TEV) cleavage of MSP, 10 mL of TEV, with 5 mM DTT, was added to the purified MSP sample. The sample was then transferred to 10 kDa dialysis tubing and dialyzed against 5 L of buffer (50 mM Tris, 300 mM NaCl, 50 mM imidazole) at 4 °C for approximately 16 h. After dialysis, the samples were subjected to Ni-NTA purification. In a similar way as above, a 5 mL Ni-NTA column was charged with $NiSO_4$ and rinsed with deionized water. The column was then equilibrated with 15 mL of buffer (40 mM Tris, 300 mM NaCl, and 50 mM imidazole). The MSP sample was applied to the column, and the flowthrough was collected as it contained cleaved MSP without the histidine tag. The MSP sample was dialyzed against 4 L of end buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA) for 6 h. After 6 h, the buffer was refreshed, and the sample was dialyzed for another 16 h. The amount of MSP after cleavage was calculated by using a spectrophotometer at 280 nm. The sample was concentrated using a 10 kDa centrifugal concentrator (Amicon) to a final concentration of 3 mg/mL. Once the desired concentration was reached, 0.01% (w/v) sodium azide was added to the MSP sample, and it was stored at −20 °C until the nanodiscs were prepared.

Expression and Purification of N-Glycosyltransferase. The expression and purification of the glycosylation enzyme *N*-glycosyltransferase (NGT) can be found in the manuscript by Ahangama Liyanage et al.[10] Briefly, the enzyme NGT was recombinantly expressed in BL21 *E. coli* as a histidine-tagged protein. Cells were harvested after 4 h of induction by L-arabinose. The cells were lysed using sonication and then centrifuged at 35,000× g. The protein was purified from the cell lysate using gravity nickel affinity chromatography. The eluent, containing NGT, was concentrated to 25 μM by using a 10 kDa centrifugal concentrator (Amicon).

Expression and Purification of γ-Sarcoglycan. The expression and purification of the membrane protein γ-sarcoglycan can be found in detailed description in the manuscript by Jamaleddine et al.[34] Briefly, full-length human γ-sarcoglycan was recombinantly expressed in BL21 *E. coli* as a fusion protein with TrpLE. Cells were harvested after 4 h of induction with IPTG. Cells were lysed using sonication, and the inclusion bodies were isolated for cleavage of the target protein from the fusion protein using chemical cleavage with cyanogen bromide. The cleaved protein was purified using size exclusion FPLC. Pure protein was dialyzed and lyophilized. Dried γ-sarcoglycan was stored at 4 °C until it was used for the formation of proteoliposomes.

Assembly of the DMPC/MSP Nanodiscs. Upon purification of MSP and the membrane protein γ-sarcoglycan, the next step was the preparation of the nanodiscs. Eight milligrams of the lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was dissolved in 1 mL of chloroform in a glass test tube and dried under nitrogen gas forming a lipid film. The film was completely dried by desiccation overnight. The lipid film was then rehydrated with 500 μL of 58.1 mM sodium cholate in water. The sample was transferred between being vortexed and being placed in a heat bath at 40 °C until the DMPC was fully dissolved. Separately, 0.3 mg of γ-sarcoglycan was dissolved in 500 μL of 80 mM *n*-dodecyl-β-maltoside (DDM) detergent. Four microliters of 1 M NaOH was added, and the sample was placed in a sonication bath to aid the solubilization of the protein. Once the lipid and the protein were dissolved in their respective solutions, the clear samples were combined and placed on a rotator at RT and left to incubate for 2 h.

Using an adaptation of methods described previously by Ritchie et al., we removed the detergent using dialysis.[32] After 2 h, the sample was transferred to 10 kDa dialysis tubing and dialyzed against 5 L of 20 mM phosphate buffer, pH 7.0. Buffer changes were made every 6 h until the clear sample turned slightly translucent, signaling the removal of detergent and the formation of proteoliposomes, as light scattering and turbidity are indicators of the existence of the large lipid assemblies.[33] At that point, 1 mL of MSP was added to the sample at a concentration of 3 mg/mL and nanodisc formation was confirmed by the sample turning clear again. The sample was then concentrated using a 10 kDa centrifugal concentrator (Amicon) to a sample volume of 500 μL.

Glycosylation of γ-Sarcoglycan. The 500 μL sample of γ-sarcoglycan in nanodiscs was separated into two 250 μL samples. One of these samples was used as a control going forward, while the other would serve as the reaction mixture. 1.4 mg of uridine diphosphate glucose (UDP-glucose) was added to the reaction mixture but not to the control. 250 μL of NGT at a concentration of 25 μM was added to both samples so each sample contained 500 μL of solution. The samples were then placed on the rotator for 24 h at RT. These samples were then dialyzed against 5 L of ddH_2O in separate dialysis containers to avoid cross-contamination. The water was changed every 6 h until the protein formed a precipitate. The samples were transferred to separate tubes and centrifuged to pellet the precipitated protein. The supernatant was discarded, and pellets were frozen and placed on a lyophilizer to dry the protein to a powder.

Mass Spectrometry of Glycosylated Samples. Following glycosylation, the dried protein powder containing MSP, NGT, and γ-sarcoglycan was prepared for gel electrophoresis. Approximately 0.1 mg of the reaction mixture was dissolved in 80 μL of LDS buffer (50 mM Tris−HCl, 50 mM Tris base, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 12.5 mM ethylenediaminetetraacetic acid, and 0.02% bromophenol blue). The protein was mechanically mixed with a 1 mL syringe, with the protein buffer solution pulled up through the needle 30 times. The sample was then boiled in a hot bath at 100 °C for 10 min.

Twenty microliters of the prepared reaction mixture was loaded onto an SDS-PAGE gel. Three microliters of PageRuler Prestained Protein Ladder (ThermoFisher) was loaded with one empty well between the ladder and the sample to avoid contamination. The samples were run on a gel at 150 V and 400 mA for 40 min. The gel was placed in Coomassie Blue stain solution (50% methanol, 40% water, 10% acetic acid, 1 g Brilliant Blue G-250) and heated in a microwave for 45 s and placed on a rocker for 1 h. After 1 h, the Coomassie Blue stain solution was removed and destain solution (50% water, 40% methanol, 10% acetic acid) was added. The gel was heated in a microwave again for 45 s in the destain solution and placed back on the rocker overnight. The gel was taken out of destain after 16 h and placed in deionized H_2O. The control sample was prepared in the same manner for trypsinolysis.

Bands that represented γ-sarcoglycan in the gel were excised and the gel slices were subjected to trypsin digestion using the standard methodologies described in Voruganti et al.[35] The trypsin-digested peptides were separated on a 75 μm × 50 cm nanocolumn (Acclaim PepMap, Thermo PN 164942) using a

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water to acetonitrile gradient with 0.1% formic acid and a gradient range of 3−30% acetonitrile over 120 min. Eluted peptides passed through a stainless-steel emitter before being ionized by a NanoSpray Flex ion source (Thermo). A "high−high" "top-speed" data-dependent acquisition, using a quadrupole-Orbitrap mass spectrometer (ThermoFusion) was performed to analyze the parent peptide ions at a resolution of 120,000 where they were selected for HCD dissociation. The resulting fragment ions were analyzed at a resolution of 30,000.

The Byonic software application (Protein Metrics) was used to identify peptides by searching RAW instrument files against the sequence of γ-sarcoglycan and the same sequence with an N-linked glycosylation (+162.0528). In addition, a database of 4306 *E. coli* protein sequences from UniProt and 9 *N*-glycosyltransferase sequences was included in the identification search. The Byonic Preview Module was used to optimize the search settings.

The quadrupole was programmed for targeted MS/MS scans of mass-to-charge ratios of 527.53, 703.03, and 1054.05 (+4, +3, and +2 ions), representing the ions of the parent peptide, residues 98−115, that contained the glycosylation site for selective reaction monitoring. HCD fragmentation was done on each selected ion, followed by wide-band scanning of fragment ions at a resolution of 60,000. Chromatograms of specific ions were extracted using parent ion scan filtering along with secondary filtering based on fragment m/z values.

AUTHOR INFORMATION

Corresponding Author

Gabriel A. Cook − *Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078, United States;* orcid.org/0000-0002-3413-8361; Email: gabriel.cook@okstate.edu

Authors

Michael S. Harris − *Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078, United States*

Rachel F. Dolan − *Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078, United States*

James R. Bryce − *Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078, United States*

Jonas G. Ewusi − *Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06135

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

LC-MS/MS, liquid chromatography−mass spectrometry/mass spectrometry; NGT, *N*-glycosyltransferase; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; MSP, membrane scaffold protein; DDM, *n*-dodecyl-β-maltoside; IPTG, β-D-thiogalactopyranoside; FPLC, fast performance liquid chromatography; LDS, lithium dodecyl sulfate

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