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Covalently circularized nanodiscs; Challenges and applications

Mahmoud L. Nasr and Gerhard Wagner

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts, USA

Abstract

Covalently circularized nanodiscs (cNDs) represent a significant advance in the durability and applicability of nanodisc technology. The new cNDs demonstrate higher size homogeneity and improved stability compared with that of non-circularized forms. Moreover, cNDs can be prepared at various defined sizes up to 80-nm diameter. The large cNDs can house much larger membrane proteins and their complexes than was previously possible with the conventional nanodiscs. In order to experience the full advantages of covalent circularization, high quality circularized scaffold protein and nanodisc samples are needed. Here, we give a concise overview and discuss the technical challenges that needed to be overcome in order to obtain high quality preparations. Furthermore, we review some potential new applications for the cNDs.

Introduction

Phospholipid bilayer nanodiscs offer a detergent-free lipid bilayer model, facilitating the studying of membrane proteins in a physiologically relevant environment[1–3]. A nanodisc is typically composed of two copies of α -helical- amphipathic proteins, termed membrane scaffold proteins (MSPs)[2,3]. The hydrophobic face of MSPs interacts with the lipid acyl chain while the hydrophilic face is located at the outside surface to allow the nanodisc to be soluble in solution. Nanodiscs are widely used for both complexes and single molecule studies of membrane protein structure and function. There are several excellent recent reviews covering nanodisc applications in studying membrane proteins [4*,5*]. While these nanodiscs have been around for over a decade, their utilities for structural and functional studies of membrane proteins have not reached their full potentials. This is due to heterogeneity of size and the number of membrane proteins enclosed, and only small nanodiscs could be constructed with the currently available protein scaffolds[6–8]. To resolve these issues and to expand the stability, applicability and size of nanodiscs, we developed methods to covalently link the N- and C- termini of newly engineered scaffold protein variants based on apolipoprotein A1 (ApoA1) scaffold protein. As a result of the covalent circularization, we produced nanodiscs with a large range of discrete sizes and

Conflict of Interest.

The authors have founded NOW Scientific Inc to allow purchase of assembled circularized MSPs. Expression plasmid are available for a nominal fee, and detailed protocols have been published.

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defined geometric shapes[9**]. Here, we give a concise overview of the technical challenges that needed to be overcome in order to obtain high quality circularized scaffold proteins and nanodiscs. In addition, we discuss the potential new applications that could be offered by these newly engineered nanodiscs.

The benefits of circularization

The linkage of the N- and C-termini of a protein via a peptide bond provides several advantageous properties including improved thermal stability [10–12] and proteolytic resistance [12–16].

Fortunately, both lipid free and lipid-bound ApoA1 have the N- and C- termini in close proximity [17**,18], thus they represent an attractive target for circularization.

Established strategies for circular protein production include the use of various intein-fusion proteins [19,20], which allow circular protein production through expressed-protein ligation or protein trans-splicing. Alternatively, circular proteins can be obtained by using sortase transpeptidases [21], or chemical ligations [22]. More recently, butelase 1 enzyme has been successfully used to circularize peptides and proteins [23].

Sortase A from *S. aureus* has several advantages that have led to its extensive use for protein site-specific modification and circularization. First, it is easy to get large quantities of the recombinant enzyme. Second, it accepts a broad variety of substrates as long as they contain the LPXTG recognition sequence (X represents any amino acid). Third, circularization using sortase leads to incorporation of only a small five-residue recognition sequence into the circularized product. There are a number of sortase A enzyme variants that can be used for protein circularization. Aside from wild-type sortase A, evolved sortases are available [24,25].

Circularization vs. multimerization of NWs by Sortase A

We have engineered four different variants of circularizable scaffold proteins: NW9, NW11, NW30, and NW50 (NW stands for NanodiscWidth) which assemble ~ 8.5, 11, 15, 50 nm nanodiscs respectively. The scaffold proteins may undergo circularization (intramolecular transpeptidation) or multimerization (intermolecular transpeptidation) followed by circularization[9]. The ratio of circularized product to multimeric product depends both on the size of the protein and on the concentration during the circularization reaction [26].

We perform the circularization reaction at lower protein concentration (less than 20 μ M) to obtain predominantly monomeric circularized products. Performing the reaction at higher concentration could result in a significant amount of multimerization byproducts (Figure 1a). Interestingly, we found that these multimerization byproducts (circularized) also form nanodiscs that can be separated to some extent by size exclusion chromatography. This observation inspired us to design new DNA constructs for making larger scaffold proteins (i.e. NW30 and NW50) [9**].

NW constructs expression and purification

All of the NW expression plasmids have been deposited at and are available from the Dana Farber/Harvard Cancer Center (DF/HCC) plasmid depository (<http://dnaseq.med.harvard.edu/>). The NW constructs contain a tobacco etch virus (TEV) protease-cleavable N-terminal His tag followed by a single glycine, and a C-terminal sortase-cleavable His tag (figure 1b). The presence of these two sites ensures covalent linkage between the N- and C- termini of NW while still preserving the function to form nanodiscs.

The expression yield per liter culture is dependent on the NW construct, media and whether a fermenter or standard shaker was used. In general, the expression of NW9 and NW11 is better than the longer NW variants (NW30 and NW50). We typically obtain around 50–80 mg of purified NW9 and NW11 from 1L LB medium in shaker flasks. On the other hand, we obtain around 7–15 mg for NW30 and only 5 mg for NW50. We observed that a significant amount of the larger NW variants remains in the insoluble fraction during cell lysis. Therefore, we solubilize the insoluble inclusion bodies in guanidine hydrochloride to recover more protein. Table 1 lists some of the issues that can arise during the expression and purification of NW proteins and offers solutions.

NW circularization

There are three methods that can be used to achieve NW circularization. These methods include circularization over copper chips, over nickel beads, or in solution. We routinely use the in-solution method as we can obtain milligram amounts of circularized proteins. Also, this method is the most cost effective one. We have provided a step-by-step protocol for the production of circularized NW proteins (cNWs) in our earlier paper [9]. The workflow for circularization and the purification is depicted in Figure 2. We dilute the NW to encourage intramolecular circularization and minimize the multimerization reaction. In general, a final NW concentration of 20 μ M or less usually resulting in more than 95% circularized monomeric products. We add sortase to a final molar ratio of 2:1, NW: sortase. The reaction is incubated at 37 °C for 2–4 hours. Next, the covalent sortase inhibitor AA EK2 [27] is added, and the solution is incubated for another 30 min at room temperature. Protein that did not undergo circularization is removed by binding to a Ni-NTA column. The cNWs are further purified by size-exclusion chromatography to remove most of the multimerization products, followed by ion exchange chromatography to eliminate the hydrolysis products and the remaining multimerization products.

Circularized nanodisc assembly: General considerations

There are several factors to consider when attempting incorporation of a membrane protein into cNDs. First, the lipid to NW ratio is very important and needs to be empirically determined in each membrane protein case in order to obtain a high yield of incorporation and a homogenous nanodisc preparation. This can be done by screening for nanodisc homogeneity and yield by size exclusion chromatography. Depending on the required surface area of a membrane protein that needs to be inserted, we subtract the corresponding number of lipid molecules from the initial lipid to NW ratio. We estimate that a single

transmembrane (TM) helix usually takes up around 70–80 Å². Thus, around 560 Å² would be required for a 7 TM-helical protein like a GPCR this would require around.

Using a suboptimal lipid amount will result in loose and unstable nanodiscs as the lateral pressure imparted by the belt protein will be abolished or significantly reduced. In this case, the lipid will be less constrained and the movement of the embedded membrane protein will not be reduced. On the other hand, using excess lipids will not be as damaging since the covalent circularization of the belt protein will not allow the nanodisc to exceed the belt's maximum diameter. In this case, the lateral pressure imparted by the belt protein will be fully utilized. One potential issue that could arise when using excess lipids is the formation of lipid aggregates and liposomes. These aggregates/liposomes are easy to separate from the smaller nanodiscs (9,11,15 nm nanodiscs), however it might become challenging to separate them from the larger ones (i.e 50 nm nanodiscs).

It is important to identify the ideal lipid composition that supports function/stability for a particular membrane protein. Often we find that choosing the ideal lipids leads to better incorporation yield.

The smallest nanodiscs (8.5, 11 nm) are the most stable and thus are well suited for NMR structural and dynamic studies. The larger size nanodiscs still provide long-term stability of the incorporated membrane protein and allow for high resolution structural studies by cryo-EM.

Potential new opportunities offered by covalently circularized nanodiscs

1- Better Vaccine

Conventional nanodiscs have shown promise in vaccine development. Several antigens including the viral protein gp160 from HIV [30] and hemagglutinin (HA) from influenza [31] have been successfully incorporated into nanodiscs. HA in nanodisc (HA-ND) vaccination induced efficient immune response and protective effect in a mouse model. Sera from HA-ND-immunized mice had significantly higher anti-HA IgG levels than HA-only immunized mice. Moreover, HA-ND induced anti-HA antibody response that was more broadly neutralizing than that induced by HA alone. Recently, ApoA1-mimetic peptide based nanodiscs have been used to develop a new vaccine system suited for personalized cancer immunotherapy [32**].

The improved stability of cND relative to conventional nanodiscs not only could have impact on the shelf life of the vaccine preparations but also could improve the antigen lifetime *in vivo*, which might be essential in some cases. Furthermore, cNDs could provide improved homogeneity and better control of the number of antigens incorporated into each nanodisc. Finally, the larger size cNDs could be very useful to incorporate several copies of antigens/ adjuvants in the same nanodisc.

2- Structural and functional studies of large membrane protein complexes in bilayer

Large cNDs provide tools to incorporate much larger membrane proteins or their intramembrane and extramembrane complexes than previous nanodisc systems have

allowed. For example, these large nanodiscs could be used to incorporate and study the various pore architectures and the membrane insertion mechanisms of pore forming toxins and proteins (PFPs). The structural studies of these PFPs could provide insights to enable the development of novel therapeutic strategies that would prevent the development of bacterial resistance. Also, large cNDs can be used to study very large complexes such as mammalian respiratory complex I which contains 45 subunits and represents one of the largest membrane-bound enzymes in the cell [33].

3- Virus entry

One of the most exciting new applications of large cND is providing enough surface area to act as a surrogate membrane for the study of the early steps in virus infection. For example, we have used 50-nm cNDs to study the question of how simple non-enveloped viruses like poliovirus (~ 30 nm) transfer their genomes across membranes to initiate infection. EM images show the poliovirus bound to 50-nm cNDs, which are decorated with the CD155 receptors. Excitingly, the EM images reveal the formation of a putative pore in the cNDs and show individual viruses ejecting RNA across membranes [9].

Currently, we are working on extending the nanodisc sizes beyond 80 nm for two reasons; first, we would like to study the viral entry of larger non-enveloped viruses. Second, we believe these very large nanodiscs (> 80 nm) will be useful in studying enveloped virus binding and membrane fusion.

Conclusions

Covalently circularized nanodiscs represent a significant advance in the durability of nanodisc technology. We discussed the technical challenges that need to be overcome to obtain high quality cND preparations and offered solutions. Furthermore, we outlined some potential new applications that could be offered by these newly engineered nanodiscs.

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- of outstanding interest

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Highlights

- cNDs represent a significant advance in the applicability and durability of nanodisc technology.
- We highlighted the technical challenges that needed to be overcome to obtain high quality cNDs.
- Large cNDs can be used to study the early steps in virus infection.

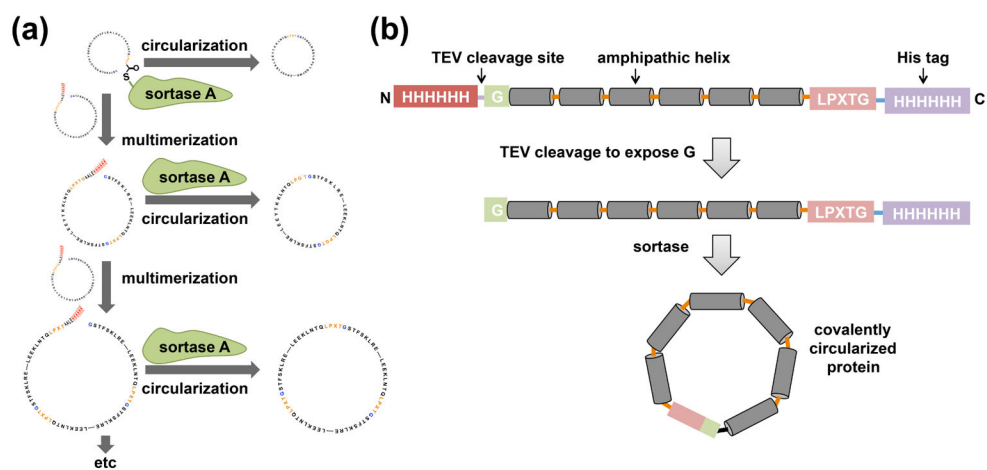


Figure 1. Circularization and multimerization of NWs by sortase A. **(a)** The addition of sortase A to a concentrated scaffold protein solution can lead to multimerization followed by circularization. **(b)** A general outline of the constructs that are used for making covalently circularized nanodiscs. This figure is adapted from reference 9.

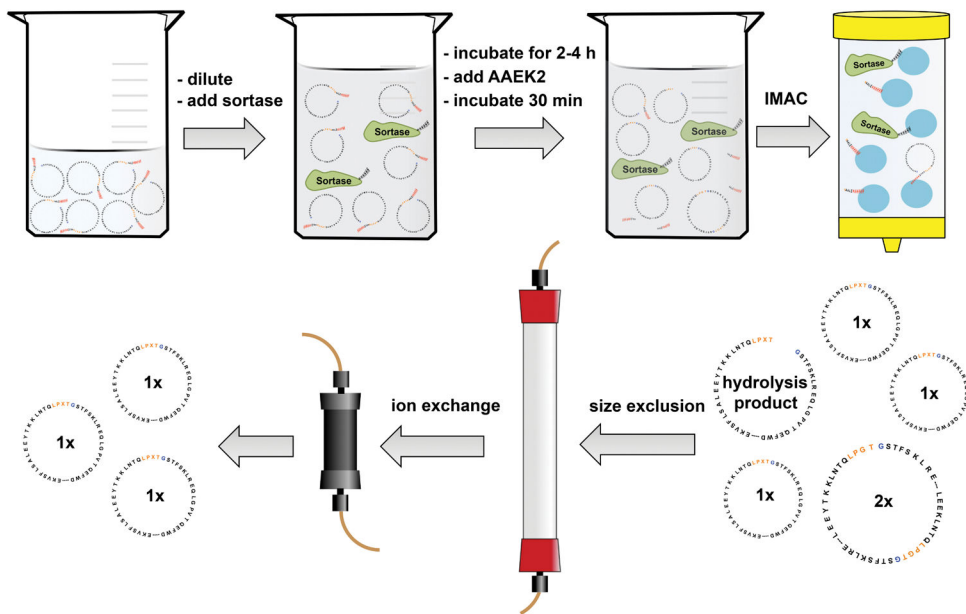


Figure 2. Schematic of the in solution method for scaffold protein circularization. After the IMAC step, cNWs are further purified by size-exclusion followed by ion exchange chromatography to remove the multimerization and hydrolysis byproducts.

Table 1

Troubleshooting table.

Problem	Possible reason	Solution
Expression and purification of NWs		
NW expression yield is low	<ul style="list-style-type: none"> Inclusion bodies formation. Prolonged post-induction growth results in decrease in NW yield. 	<ul style="list-style-type: none"> If you have inclusion bodies then follow the protocol for the purification of the insoluble fraction as described by Nasr et al. [9] Make sure you do not exceed 5 hours post-induction time at 37°C.
NW purity is not good after His purification	<ul style="list-style-type: none"> Non specific binding to Ni beads. 	<ul style="list-style-type: none"> Use 30–40 mM imidazole in the last wash step instead of 20 mM. Keep in mind that NW constructs contain 2 His tags and should bind more tightly to Ni beads. Try gradient elution.
Removal of the N terminal His tag by TEV is not complete	<ul style="list-style-type: none"> TEV amount used is not enough. TEV activity is low. Incubation time or temperature is not ideal. Autolysis of TEV. Buffer is not ideal. 	<ul style="list-style-type: none"> Use more TEV and/or longer incubation. Perform TEV cleavage at a higher temperature 20–34°C. Autolysis of TEV protease can be avoided by using more stable TEV mutant such as S219V mutant [28] or S219N [29].
NW precipitates during TEV cleavage	<ul style="list-style-type: none"> Salt concentration is low. 	<ul style="list-style-type: none"> Increase salt concentration to 200 mM or more.
Circularization of NW		
Circularized reaction is proceeding very slowly or incomplete.	<ul style="list-style-type: none"> Sortase activity is low. Incubation time and temperature is not ideal Buffer is not ideal TEV removal of the N terminal His tag is not complete. 	<ul style="list-style-type: none"> Use fresh sortase if possible. We noticed that sortase activity decreases significantly after 3–4 weeks at –80°C. Make sure that TEV cleavage is complete before proceeding to circularization step.
Formation of hydrolysis products	<ul style="list-style-type: none"> pH is not ideal. Usually pH higher than 7.5 results in more hydrolysis products. Too long incubation with sortase. Sortase is not removed completely. 	<ul style="list-style-type: none"> Make sure pH does not exceed 7.5. Lower pH to 6–7. In this pH range the circularization reaction will take longer time to complete but will result in less hydrolysis products. Use HPLC or ion exchange to eliminate hydrolysis products. Use excess Ni beads to make sure sortase is completely removed after circularization. Add 20 mM EDTA after the reverse Ni step to make sure sortase is completely inactivated (in case you still have traces of sortase in the final product).