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# Covalently circularized nanodiscs; challenges and applications

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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.

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## 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-terminus to the C-terminus 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 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-terminus and C-terminus 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-terminus and C-terminus 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 *Staphylococcus 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 versus 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 μM) 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/).

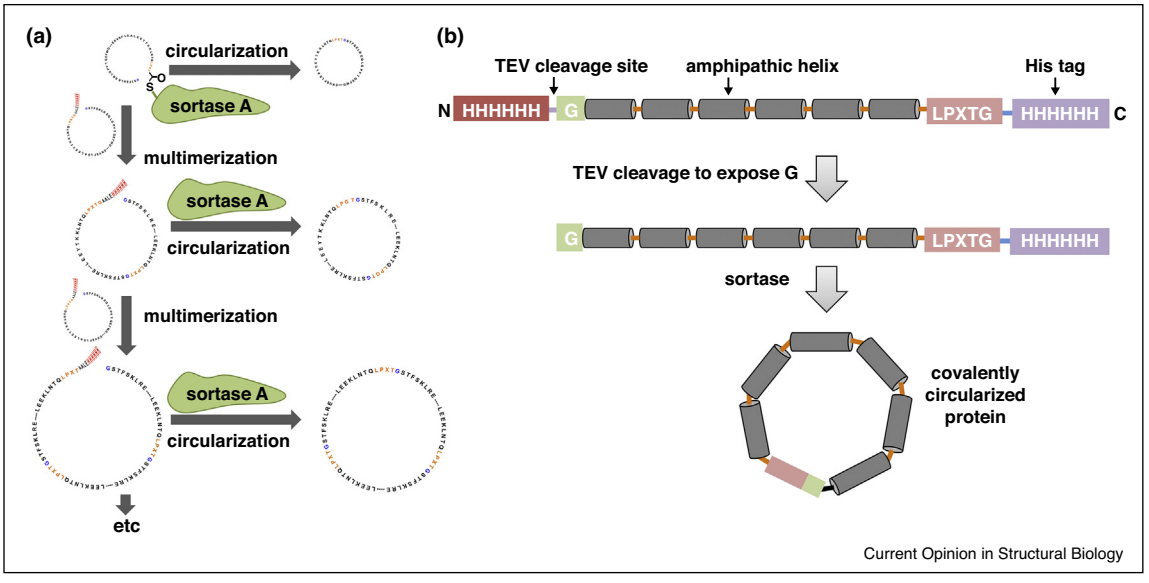
The NW constructs contain a tobacco etch virus (TEV) protease-cleavable N-terminal His tag followed by a single glycine, and a C-terminal sortase-cleavable His tag (Figure 1b). The presence of these two sites ensures covalent linkage between the N-terminus and C-terminus of NW while still preserving 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

**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 Ref. [9••].