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Nanodisc assembly from bacterial total lipid extracts

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ABSTRACT

Nanodiscs are discoidal lipoproteins that have often been used as vehicles to study membrane proteins in their native configuration. Nanodiscs have been primarily made from synthetic lipids. However, nanodiscs also offer a format by which native lipids can be studied in their natural configuration. Here, we present a method to synthesize nanodiscs from bacterial total lipid extracts using the biothreat agent, *Yersinia pestis,* as a proof-of-concept. The creation of nanoparticles entirely composed of bacterial lipids supports membrane characterization and vaccine antigen discovery without the inherent safety concerns associated with live bacterial cells of this Tier 1 select agent pathogen.

1. Introduction

Nanodiscs, or nanolipoproteins, are synthetic lipid bilayers bound by membrane scaffold proteins (MSPs) that form a discoidal structure between 8 and 16 nm in diameter (Denisov and Sligar, 2016). One of the most common applications of nanodiscs is to use them as vehicles to study transmembrane proteins that only fold into their native structure when incorporated in a lipid bilayer (Denisov and Sligar, 2016). More recently, nanodiscs have been used in applications such as diagnostics (Bariwal et al., 2022), vaccine development (Kuai et al., 2018; Guo et al., 2023), and cancer immunotherapy (Kuai et al., 2017). In the realm of vaccine development, nanodiscs are a potential platform for presenting lipid and amphiphilic antigens in their natural conformation (Lenz et al., 2022). For example, the amphiphilic bacterial molecules lipopolysaccharide (LPS), lipoteichoic acid (LTA), and lipoarabinomannan (LAM), are well known toll-like receptor (TLR) agonists that stimulate the host immune system (Babolewska et al., 2012; Stromberg et al., 2018). In theory, these amphiphilic molecules could be incorporated into nanodiscs as integrated lipids in a membrane-like environment. It is also possible that other immunogenic lipids exist but have not been identified because of the challenges their biochemistry creates in purifying, characterizing, and presenting them in a lipid-compatible vaccine platform. Nanodiscs offer a potential way to circumvent this challenge by providing an environment that is favorable to lipid and amphiphilic molecules. Previously, nanodiscs have been created using *Pseudomonas aeruginosa* outer membrane vesicles (Noh et al., 2022). However, outer membrane vesicles (OMVs) are difficult to purify and are not produced by all bacteria. Here, we describe a protocol to form nanodiscs using entire bacterial lipid extracts that can be applied to study membrane components of other bacterial pathogens.

In this work, we adapted previously-published methods on synthetic nanodisc formulation to create nanodiscs comprised of bacterial membrane total lipid extracts encased by membrane scaffold protein 1D1 (MSP1D1) (Denisov et al., 2004). MSP1D1 is an amphipathic scaffold protein derived from apolipoprotein A-1 and forms nanodiscs of approximately 9–10 nm in diameter (Ritchie et al., 2009; Bayburt et al., 2002). We chose to use the Gram-negative bacterial pathogen *Yersinia pestis* as a proof-of-concept because it is classified in the highest risk category of potential biothreat agents and there is no licensed vaccine available (Sun and Singh, 2019). Other researchers have formulated nanodiscs that incorporate LPS and/or the surface protein Ail from *Y. pestis* into synthetic lipid nanodiscs to study protein structure and function (Chandan Singh et al., 2020; Dutta et al., 2017). To our knowledge, this is the first time nanodiscs have been made from bacterial total lipid extracts without the addition of any synthetic lipids. These methods could be applied to broadly interrogate the lipid membrane of other bacterial pathogens of interest for antigen identification.

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2. Methods and results

The overall workflow for nanodisc assembly from bacterial total lipid extracts is described in Fig. 1.

2.1. Bacterial growth

Y. pestis strain A1122 (NR-636, BEI Resources), a strain that is excluded from select agent criteria, was streaked on tryptic soy agar (TSA, VWR 100217–300) plates and incubated at 28 °C for 24–48 h. Individual colonies were isolated and grown in sterile tryptic soy broth (TSB, BD Bacto 211825) at 28 °C for 24–48 h at 150 revolutions per minute (RPM) (Fig. 1A). The bacterial cultures were grown to stationary phase as determined by OD 600 measurements and pelleted by centrifugation at 4 °C and 5000 relative centrifugal force (RCF) for 10 min. The supernatant was removed, and the bacterial pellet was stored at 4 °C.

2.2. Total lipid extraction

A modified Bligh & Dyer lipid extraction method was used to extract lipids from the bacterial pellet (Fig. 1A) (Bligh and Dyer, 1959). First, 3.75 mL of a 1:2 (v:v) ratio of chloroform (Sigma 132950):methanol (Sigma 179337) was added to 1 g of the bacterial pellet, and the sample was vortexed for 10–20 sec to bring the pellet into solution. An additional 1.25 mL of chloroform was added to the solution and mixed by pipetting up and down. Next, 1.25 mL of nanopure water was added and the solution was vortexed again until it appeared milky. The solution was centrifuged at 1000 RCF for 5 min at room temperature to yield a two-phase system consisting of an aqueous top layer and an organic bottom layer. The organic phase containing bacterial lipids was recovered manually by pipette (typically ~1 mL). Lipid extracts were stored at –20 °C. Non-viability tests were performed on each lipid extract by plating 10 % of the volume onto sterile TSA plates and incubating for a week at 28 °C. Plates were inspected by eye and were deemed non-viable if no growth was present.

Table 1
Optimal molar ratio for synthetic lipids converted to mass ratio.

Molar Ratio (MSP1D1:Lipid)	Synthetic Lipid	Molar Mass Synthetic Lipid (g/mol)	Mass Ratio
1:70	DMPG	666.9	1:1.89
1:30	DOPC	734.039	1:0.92
1:90	DPPC	734.039	1:2.68
1:65	POPC	760.091	1:2.00
1:80	DOTAP	663.1	1:2.15

DMPG, dimyristoylphosphatidylglycerol; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DPPC, Dipalmitoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; DOTAP, 1,2-Dioleoyl-3-trimethylammonium.

2.3. Nanodisc formulation

Previous work with synthetic lipids has shown that both the molar ratio of MSP:lipid and the transition temperature of lipids are important criteria in the success of nanodisc formulation (Denisov et al., 2004). In this work, the molecular weight of *Y. pestis* total lipid extracts was unknown, so a mass ratio was used. In order to determine a range of mass ratios to test, published molar ratios of MSP1D1:synthetic lipids were converted to mass ratios (Table 1) (Denisov et al., 2004; Bayburt et al., 2002). The maximum, minimum, and median of this range were used to guide nanodisc formulation with MSP1D1 and *Y. pestis* total lipid extracts (Fig. 1B). The following mass ratios were tested: 1:2, 1:0.92, 1:2.68. Transition temperatures of synthetic lipids were reviewed, and the following temperatures were tested: 25 °C, 37 °C, 40 °C (Denisov et al., 2004; Bayburt et al., 2002).

Bacterial total lipid extracts suspended in chloroform were dried overnight in a biosafety cabinet, massed, and resuspended in cholate buffer (26 mM sodium cholate (Sigma C6445)) to a final lipid

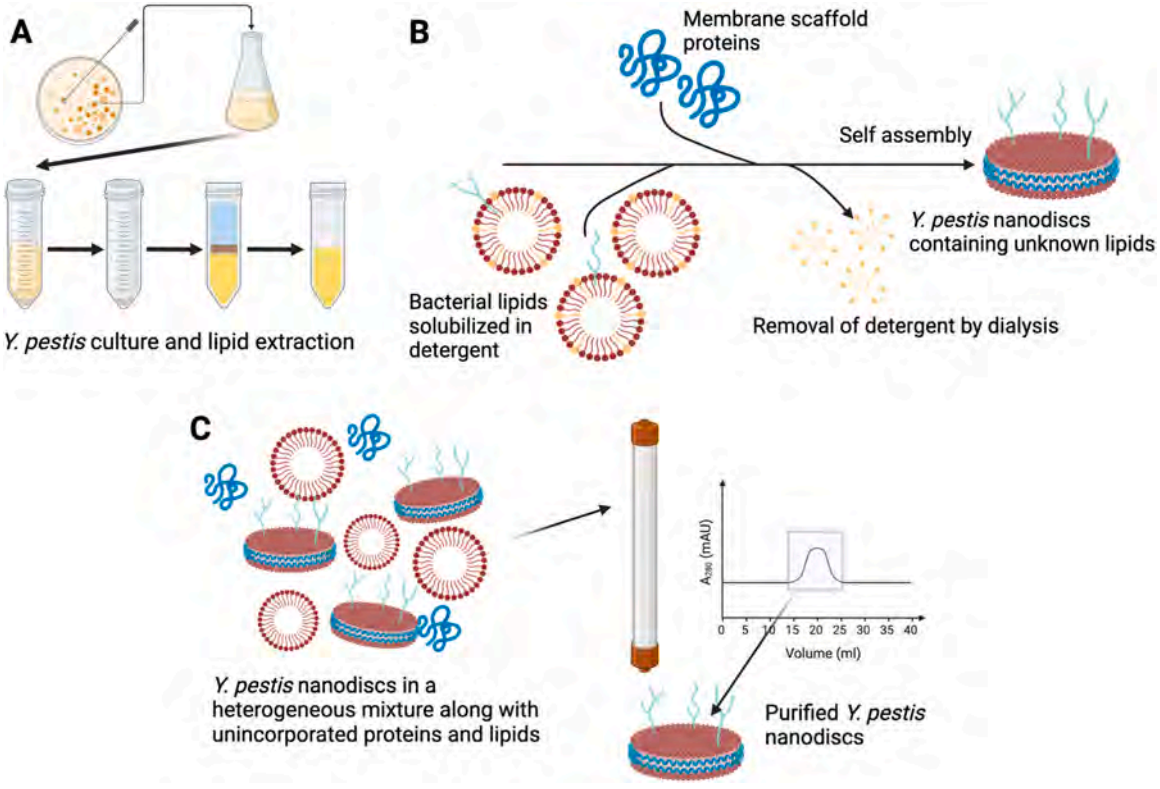

Fig. 1. Experimental protocol for nanodisc formulation. A) *Y. pestis* culture and extraction of lipids; B) nanodisc formation using extracted *Y. pestis* lipids by self-assembly; C) purification of *Y. pestis* nanodiscs by size exclusion chromatography (SEC) to separate unincorporated proteins and lipids from nanodiscs.

concentration of 10 mg/mL. Lipids were sonicated for 15 min in a bath sonicator at 25 °C and vortexed as needed to bring the lipids into solution. They were then moved to an orbital shaker for 30 min at room temperature.

Total lipid extracts solubilized in cholate buffer were combined with MSP1D1 (Sigma MSP01, 5 mg/mL) in a glass tube to a final volume of 150 or 300 μL at the specified mass ratio being tested. The three incubation temperatures were tested (25 °C, 37 °C, 40 °C) for each ratio by incubating in a thermocycler for 1 h. After incubation, samples of the same experimental conditions (mass ratio and temperature) were combined in dialysis cassettes (Sigma PURD60100, Molecular Weight Cut-off 6–8 kDa) and dialyzed at room temperature in detergent-free (DF) buffer (Tris-HCl, 100 mM NaCl, pH 8). DF buffer was replaced three times over 24 h. Samples were removed from the dialysis cassettes and analyzed by dynamic light scattering (DLS; Malvern Zetasizer Nano ZS90) to determine particle size.

Initially, nanodisc detection by DLS was masked (data not shown), presumably due to the presence of larger lipid particles and/or aggregates, so a centrifugation step was added after dialysis to separate larger lipid particles (pellet) from the smaller nanodiscs in the supernatant. Two different biological replicates (S1 and S2) of lipid extracts were made as described above and nanodisc formulation with three centrifugation protocols was tested: no centrifugation (samples S1–1 and S2–1), 12,500 rpm for 10 min at 15 °C (samples S1–2 and S2–2), and 11,300 rpm for 45 min at 15 °C (samples S1–3 and S2–3) (N=6). Extracting lipids from different biological samples helped to account for variability between different *Y. pestis* colonies. The 45 min centrifugation step removed many of the impurities seen directly after dialysis (Appendix B, Figures S1 and S2). There was little difference between the no centrifugation and 10 min centrifugation sample sets (Appendix B, Figures S1 and S2). After testing these parameters, we found that a 1:2 MSP:lipid mass ratio, 37 °C incubation temperature, and a post-dialysis centrifugation step of 11,300 rpm for 45 min at 15 °C yielded nanodiscs of the expected ~9–12 nm size range (Fig. 2).

2.4. Nanodisc purification

Samples were purified using size exclusion chromatography (SEC) (Superdex 200 Increase 10/300 GL) controlled by an AKTA Pure 25 system (Cytiva) to separate assembled nanodiscs from other lipid and protein complexes (Fig. 1C). Running buffer was membrane scaffold buffer (20 mM Tris–HCl, 100 mM NaCl, 0.5 mM EDTA, pH 7.4, filter sterilized) and experiments were performed at room temperature. Fractions were collected in 1 mL fractions and pooled according to A280 peaks which are labeled on the chromatogram (Appendix B, Figure S3).

Fractions from the same peak were combined and analyzed by DLS for size. Of the six purified samples, each contained nanodiscs of the expected size range (~9–12 nm) in one or two of the chromatogram peaks (Appendix B, Figure S1, red boxes). In some samples, larger particles were detected via DLS post-purification by SEC (Appendix B, Figure S1). This is likely due to the propensity of heterogeneous lipid/protein assemblies to aggregate and modify without the presence of a detergent (McLean et al., 2018). Samples contained approximately 30 % nanodisc by number. In Fig. 2, DLS is reported as a measurement of % Number rather than % Volume. Because the nanodiscs are relatively small, any larger moiety in solution with them would shield their % Volume reading, regardless of the amount of larger particles present (Appendix B, Figure S1 and S2) (Babick, 2020). Fractions that elute before and after the fraction containing nanodiscs show a range in particle size from 1 to 100 nm (Appendix B, Figure S1 and S2).

Fractions were analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix B, Figure S4) to show that MSP1D1 was incorporated into fractions containing nanodiscs. Results show that the only protein present in any fraction is MSP1D1, and it is at the expected molecular weight.

Cryogenic transmission electron microscopy (Cryo-TEM) was performed by Creative BioStructure (Shirley, NY, USA) to understand the morphology and size distribution of the particles (Fig. 3). Particles appeared homogeneous in size ranging from 6 to 12 nm in agreement with DLS and SEC data with varying particle orientation (ie. face up versus side up).

3. Conclusions

DLS, SEC, SDS-PAGE, and cryo-TEM confirm the reproducible formation of nanodiscs from independent total lipid extracts derived from the bacterial pathogen *Y. pestis*. DLS and SEC were both used to confirm that these nanodiscs are of the expected size range (9–12 nm). The various peaks observed in SEC can be attributed to different shapes and densities of the particles, which are detected in the mobile phase of chromatography (Skoog et al., 2006). Due to the assembly with a heterogeneous mixture of lipids, complexes of different densities that form with MSP-1D1 may elute earlier than expected because residence time in the stationary phase of the resin can be affected by density (Skar-Gislinge et al., 2010). This information, along with our DLS and cryo-TEM data, provides evidence of nanodiscs synthesized at the expected size range (9–12 nm).

The efficiency of nanodisc formulation from bacterial lipids relies on the identification of optimal MSP:lipid ratio, incubation temperature, and centrifugation time. This work provides a template for how to develop and characterize nanodiscs from bacterial pathogens. Future work will include characterization by mass spectrometry to define the

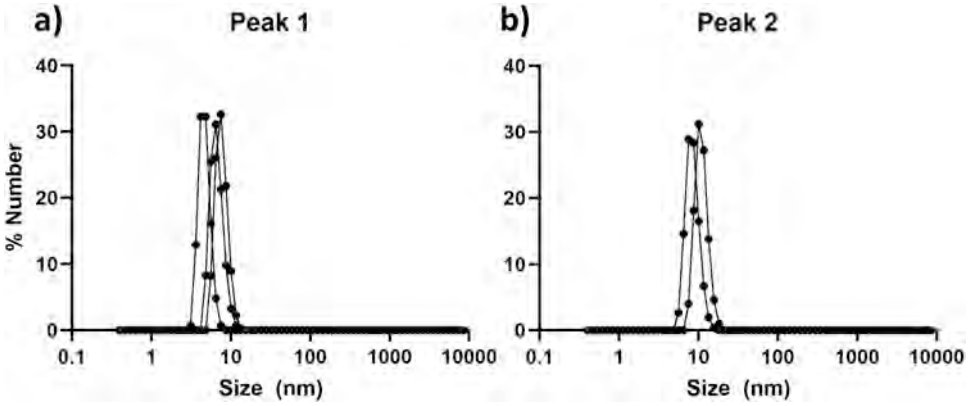

Fig. 2. Representative DLS results from sample S1–3 which was formulated using the optimized protocol of a 1:2 MSP:lipid mass ratio, a 37 °C incubation temperature, and a post-dialysis centrifugation step of 11,300 rpm for 45 min at 15 °C. Peak 1 (a) and Peak 2 (b) correspond to SEC chromatogram peaks (Appendix B, Fig. S3). Both contain nanodiscs of the expected size range, 9–12 nm. Measurements were performed in triplicate.

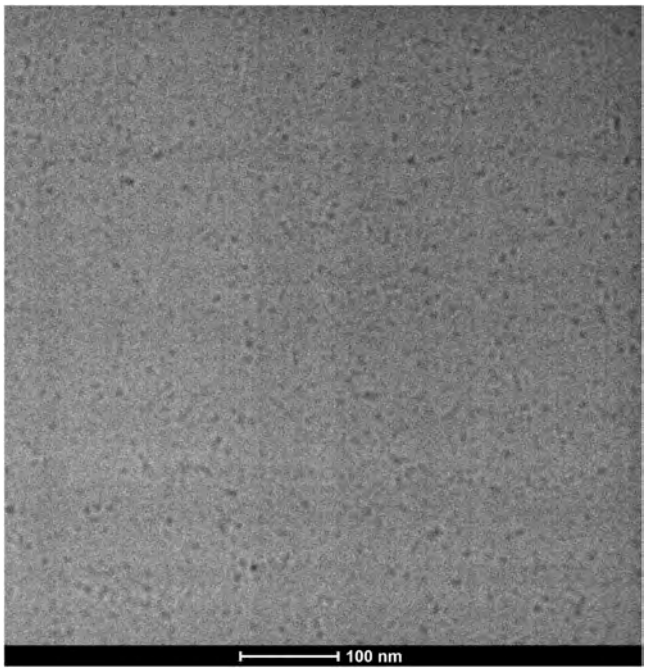

Fig. 3. FEI Talos F200C cryogenic transmission electron microscopy (Cryo-TEM) image of *Yersinia pestis* lipid nanodiscs. In collaboration with Creative BioStructure (Shirley, NY, USA), the FEI Talos F200C Cryo-Transmission Electron Microscope was used to analyze one nanodisc sample. A 5 μL aliquot of undiluted nanodiscs at 1.46 mg/mL was placed on a thin copper grid that had been glow discharged and loaded into the freezing chamber (0–5 °C) under humidity control (100 %). After blotting for 3 sec with filter paper, the specimen was vitrified with cryogen, liquid ethane cooled by liquid nitrogen. The prepared grid was used for imaging and analysis.

complete profile and concentration of lipids contained in the nanodiscs. This analysis would also provide information on the lipid types that are excluded from nanodisc formation, which may be of immunological importance. This work supports the characterization and identification of previously unknown bacterial membrane-derived amphiphilic or lipid molecules for vaccine and therapeutic development.

CRediT authorship contribution statement

Kiersten D. Lenz: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Makaela M. Montoya:** Writing – review & editing, Methodology, Investigation. **Jessica Z. Kubicek-Sutherland:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Trent R. Llewellyn:** Writing – original draft, Methodology, Investigation, Formal analysis. **Olivia R.C. Pimentel:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data is included in the manuscript and supplemental information.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.chemphyslip.2024.105425.

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