Detailed Evaluation of Nanodisc Libraries as an In Vitro Model for Synaptic ADDL Binding

Undergraduate Researcher
Dessi S. Moneva
Purdue University, West Lafayette, IN

Faculty Mentor
William L. Klein
Department of Neurobiology and Physiology
Northwestern University

Postdoctoral Mentor
Kyle C. Wilcox
Department of Neurobiology and Physiology
Northwestern University

Abstract

This paper advances an innovative method for studying the effects of amyloid β-derived diffusible ligands (ADDLs), soluble neurotoxic oligomers that are widely believed to target synaptic membranes in Alzheimer's disease (AD). Using lipid-protein nanodiscs, a synaptic membrane protein library was created to model cellular ADDL binding in vitro. This project aimed to determine the validity of such a library as a model for biological membranes, toward the ultimate goal of elucidating the interaction mechanism between ADDLs and the membrane. Biotinylation of plasma membranes and resulting nanodiscs showed that nanodiscs efficiently sample the synaptic membrane. Western blot experiments and an ADDL binding assay indicated that two different detergents used to prepare nanodiscs, sodium dodecyl sulfate and N-dodecyl-β-D-maltoside, have different protein incorporation profiles, and that use of the latter may improve protein integrity within nanodiscs. Finally, western blot and binding data suggested that the Prion protein (PrP) does not act as a direct ADDL receptor on the basis of PrP antibody blockade of ADDL binding. The work presented here has significant implications for future AD diagnostics and therapeutics.

Introduction

Alzheimer's disease (AD) is a degenerative neurological condition that causes irreversible memory loss and failure to form new memories in both early- and late-onset cases.[1,2] Despite the increasing prevalence of the disease due to an aging population, there are currently no effective molecular diagnostics or therapeutics for early AD (i.e., in living patients).[3] It is now widely accepted that dementia is caused by a neurotoxin that arises from amyloid β (Aβ), a naturally occurring peptide of 40–42 amino acids.[4–6] It is hypothesized that Aβ has two independent aggregation pathways leading to the formation of either insoluble amyloid fibrils or soluble oligomers. The soluble oligomers with toxic activity, known as amyloid β-derived diffusible ligands (ADDLs), are absent in healthy individuals. Early in AD progression, ADDLs act as gain-of-function ligands that target the neuronal synapse and initiate a toxic pathway that disrupts the ability of neurons to communicate with each other.[6]

The mechanism by which ADDLs initiate neuronal damage is poorly understood; however, there is evidence for a protein-based receptor-mediated mechanism in which ADDLs act as extracellular ligands.[7,8] Further study of this interaction between ADDLs and the hypothesized receptor is needed because the interaction is a promising pharmaceutical target. Discovery of the receptor would also allow for a better understanding of the downstream biological pathways initiated by ADDL binding.

Identifying and characterizing receptors for known ligands presents an obstacle because membrane proteins are, by nature, highly insoluble and aggregate in aqueous solution; therefore, analysis using conventional techniques is particularly difficult. Many tools, including micelles and liposomes, have been used to solubilize and study membrane proteins, but these methods have significant limitations, including a large and complex structure and inability to reproduce individual protein-protein interactions. An in vitro tool without such limitations would be particularly useful in studying the hypothesized ADDL receptor and the role of ADDLs in AD.

Background

Nanodiscs are a novel technology for stabilizing insoluble membrane proteins in solution. These self-assembling lipid-protein nanoparticles consist of a phospholipid bilayer surrounded by a belt of membrane scaffold proteins (MSPs) and an embedded membrane protein of interest.[9] MSPs are helical amphiphilic peptides derived from apolipoprotein A-I, a protein in the human bloodstream essential for the formation of high-density lipoprotein (HDL). Each nanodisc is solubilized by two MSP molecules (Figure 1).

Nanodiscs form spontaneously from their detergent-solubilized components upon the removal of detergent from the mixture. Proper formation is highly sensitive to the use of an optimal lipid:MSP ratio. Due to this relationship, genetically modified MSPs of various lengths produce nanodiscs of different diameters. Furthermore, the number of incorporated target proteins is limited by MSP length and the lipid:MSP ratio.[9] The number of target proteins captured per nanodisc can be tuned based on starting material concentrations.[10]

As a promising tool in the study of membrane proteins, nanodiscs offer numerous advantages over previous methods. Four features are of particular utility in this project. First, the nanodisc structure closely simulates the physiological environment of the plasma membrane, which allows for proteins to retain their function within nanodiscs. Second, the simplicity of nanodiscs allows the study of specific interactions between membrane receptors and their native ligands in vitro. Third, the small size and monodispersity of nanodiscs allows for the study of a limited number of target proteins, unlike micelles and

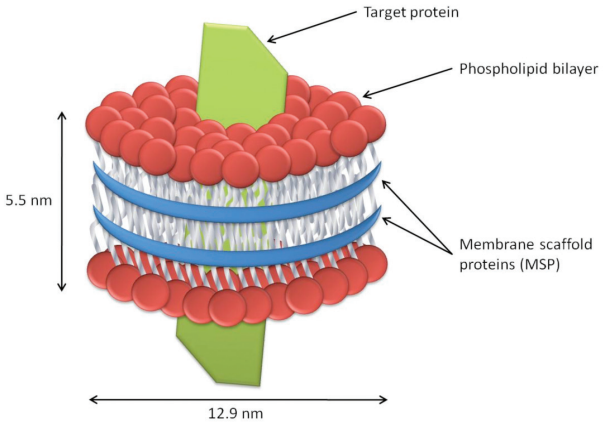

Figure 1. Nanodisc structure: lipids are shown in red, membrane scaffold proteins in blue, incorporated protein in green.

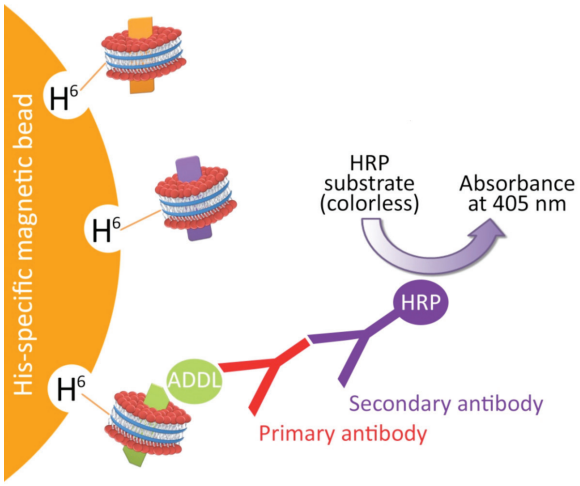

Figure 2. Colorimetric assay used to quantify ADDL binding to immobilized nanodiscs.

liposomes, which can house dozens of proteins.[11] This property is significant because separating synaptic proteins with complex interactions should improve the functional understanding of individual proteins. Finally, the MSPs can be genetically modified with various tags that allow for the immobilization of nanodiscs for use in assays. Based on these and other advantages, nanodiscs have been extensively characterized and used to study a diverse variety of membrane proteins, including functional receptors.[9–11]

Approach

Synaptic Membrane Protein Library

This paper proposes an innovative strategy for the use of nanodiscs. Rather than being created from a single purified protein, nanodiscs can be made from a heterogeneous sample of tissue-derived synaptic plasma membrane proteins, including a wide variety of synaptic receptors and ADDL receptor candidates. The result is called a synaptic membrane protein library (SMPL). Prior data show that SMPLs can reproduce the major characteristics of cellular ADDL binding; therefore, this technique is a promising method that can be applied toward the goal of ADDL receptor discovery. This project tests the capacity of SMPLs to realistically model a biological membrane in terms of receptor content and binding characteristics.

SMPL Construction

Synaptic plasma membrane proteins were isolated from rat cortex using a protocol established by Jones and Matus.[12] This membrane material was solubilized using either 0.1% sodium dodecyl sulfate (SDS) or 0.1% dodecyl-β-D-maltoside (DβM) in phosphate buffered saline (PBS). The proteins were then incubated with a 130:1 mixture of either 20 mM cholate- or 10 mM deoxycholate-solubilized palmitoyloleoyl phosphatidylcholine (POPC) lipids with the 32.6 kDa membrane scaffold protein 1E3D1 (MSP1E3D1). Upon overnight detergent removal via absorbent Amberlyte XAD-2 beads (Sigma) at 4°C, nanodiscs with a diameter of 12.9 nm were formed with individually incorporated membrane proteins. Empty nanodiscs were also formed as a control via detergent removal from an aliquot of the lipid-MSP mixture prior to the addition of detergent-solubilized membranes. Nanodiscs were purified using a nickel-nitriloacetic acid column (Qiagen) and concentrated by centrifugation through a fractionation column. Biotinylation experiments were performed using a variation of this SMPL construction in which synaptic plasma membranes were treated with sulfosuccinimidyl 6-(biotinamido) hexanoate (Sulfo-NHS-LC-Biotin) using a Pierce protocol to label exposed proteins prior to nanodisc formation.

Colorimetric ADDL Binding Assay

The capacity of SMPLs to reproduce the essential characteristics of the parent membrane, including receptor content and ADDL binding, was experimentally assessed using a colorimetric ADDL binding assay. This assay exploits MSPs that are modified with a His-tag, which allows for immobilization of nanodiscs on His-specific magnetic dynabeads (Invitrogen). Following immobilization, nanodiscs were treated with ADDLs for 90 min in F12-BSA buffer. Bound ADDLs were detected using NU-2 primary antibody, followed by a horseradish peroxidase (HRP)–linked antimouse secondary antibody. Samples were exposed to colorimetric HRP substrate (BioRad) for 5 min, and ADDL binding was quantified using absorbance at 405 nm (Figure 2).

Results

Protein Incorporation

The capacity of nanodisc libraries to efficiently sample the synaptic membrane was tested by biotinylating synaptic plasma membranes and probing the resulting nanodiscs for biotinylated proteins. Western blots of the biotinylated membrane proteins and resulting nanodiscs made with SDS were then performed to determine the extent and specificity of protein incorporation in the nanodiscs. Figure 3 shows that a vast majority of synaptic membrane proteins were efficiently transferred into nanodiscs.

Detergent Comparison

Since detergent must be used to solubilize membranes prior to nanodisc formation, the specific detergent used can have important implications for the structural and functional properties of the proteins within the resulting nanodiscs. Previously, SMPLs have been created using SDS, a harsh detergent, to solubilize the membrane proteins. The literature

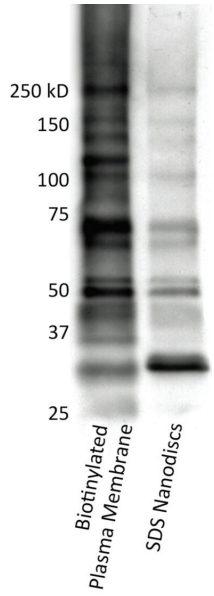

Figure 3. Western blot of biotinylated plasma membrane and resulting nanodiscs, made using SDS, showing that a vast majority of synaptic membrane proteins are efficiently incorporated into SDS nanodiscs.

indicates that a milder detergent, DβM, has been successfully used to prepare functional nanodiscs incorporating the β2-adrenergic receptor and may be a better alternative to SDS.13 Therefore, this project compared SMPLs created using either SDS or DβM and probed for the incorporation of several known receptors involved in ADDL binding, including Prion protein (PrP), insulin receptor (IR), and glutamate receptor (GluR2).

Western blot data showed that both detergents incorporated these three receptors in nanodiscs and that each detergent had a different incorporation profile, shown in Figure 4a. Preliminary data suggested that the use of SDS incorporated more protein than DβM. However, this trend was not reflected in ADDL binding assays, shown in Figure 4b, suggesting that proteins lose their native activity upon incorporation into SDS nanodiscs. It is interesting to note that DβM nanodiscs displayed higher ADDL binding than SDS nanodiscs. Despite unusually high background in the empty nanodisc control, which indicated a problem with this specific nanodisc preparation, this data may suggest that DβM is a better detergent candidate than SDS for reconstituting synaptic ADDL binding sites in nanodiscs.

Response to Hypothesized Receptor

In the literature PrP has been proposed as the ADDL receptor, a hypothesis based in part on cellular data showing that several anti-PrP antibodies block ADDL binding to varying extents according to the PrP epitopes recognized.[14] To better understand this apparent competition between PrP antibodies and ADDLs, and to attempt to reproduce and expand upon these findings, an in vitro experiment using nanodiscs was performed. This experiment also aimed to test whether the receptor organization in nanodiscs is the same as in neuronal membranes. SDS plasma membrane nanodiscs were incubated with three different PrP antibodies (7D9, 8G8, and 6D11) prior to ADDL treatment, and ADDL binding was quantified using colorimetric HRP assay.

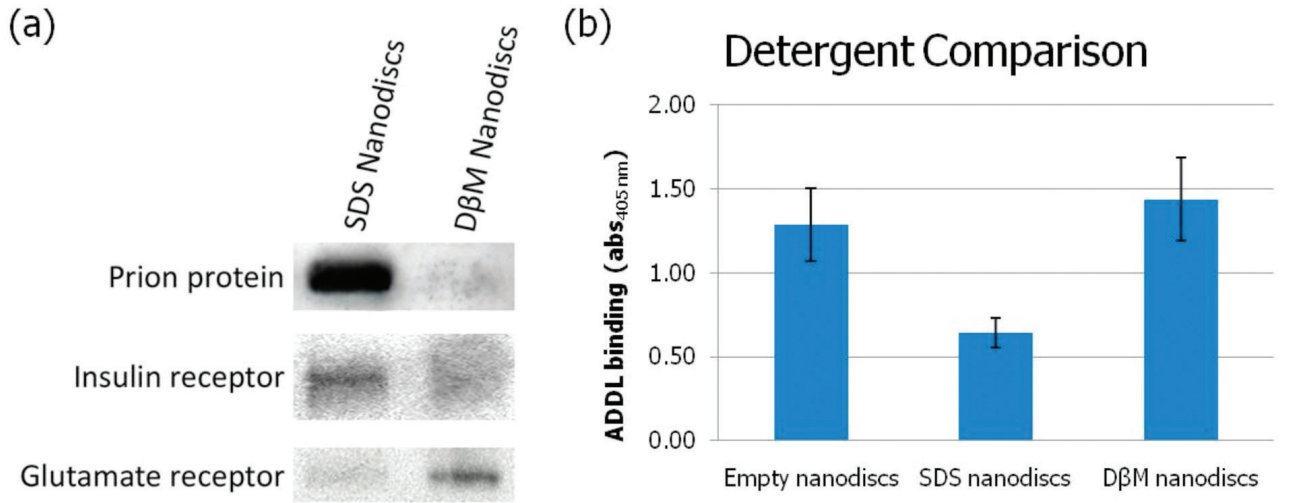

Figure 4. (a) Western blots of proteins related to ADDL binding show that SDS and DβM have different protein incorporation profiles. (b) ADDL binding results suggest that DβM nanodiscs improve ADDL binding; however, unusually high binding in empty nanodiscs indicates a problem with this particular set of nanodiscs.

It is interesting that none of the three tested PrP antibodies had a significant effect on ADDL binding in nanodiscs (Figure 5a). This result did not reflect the cellular binding characteristics published in the literature. To attempt to explain these findings, a western blot was performed on proteins released from the nanodiscs by boiling for 5 min at 100 °C. Despite similar concentrations of PrP in each of the plasma membrane nanodisc samples, as shown in Figure 5b, the three PrP antibodies were significantly different in their binding affinities for PrP in nanodiscs, as shown in Figure 5c.

Discussion

Based on this preliminary study, a novel application of nanodiscs to sample biological membranes was shown to efficiently incorporate synaptic membrane proteins, including several known receptors related to ADDL binding. Furthermore, based on ADDL binding data, DβM showed promise of being a better detergent than SDS in reproducing ADDL-receptor behavior in nanodiscs. These findings provide promising evidence that SMPLs are an effective tool for modeling biological membranes.

The data also suggest that PrP is unlikely to be a direct ADDL receptor. It is important to note that one of the PrP antibodies (6D11) binds with high affinity to nanodiscs but does not decrease ADDL binding. This evidence suggests that antibody blockade of PrP does not reduce ADDL binding and, by extension, that PrP is not acting as an ADDL receptor in nanodiscs. Since the binding affinities of the PrP antibodies to nanodiscs mimicked their ability to block ADDL binding in cells, these results may explain the published trend and implicate a non-epitope-specific effect of PrP antibodies on cellular ADDL binding. Recently, Laurén et al. have suggested that PrP may play a role in concentrating ADDLs near the receptor and therefore have a concentration-dependent effect on ADDL binding.[15]

This project should be further developed to prove that nanodiscs are a reliable model for synaptic ADDL binding. Future work should include expanding membrane biotinylation studies to include protein incorporation in DβM nanodiscs, repeating the ADDL binding assay to compare SDS and DβM, testing for incorporation of other proteins in both SDS and DβM nanodiscs, and testing other detergents as well as combinations of detergents. Moreover, it is important to test protein integrity (i.e., the ability to bind physiological or pathological ligands) within nanodiscs by performing functional assays using well-understood receptors, such as the insulin receptor and β_2-adrenergic receptor.

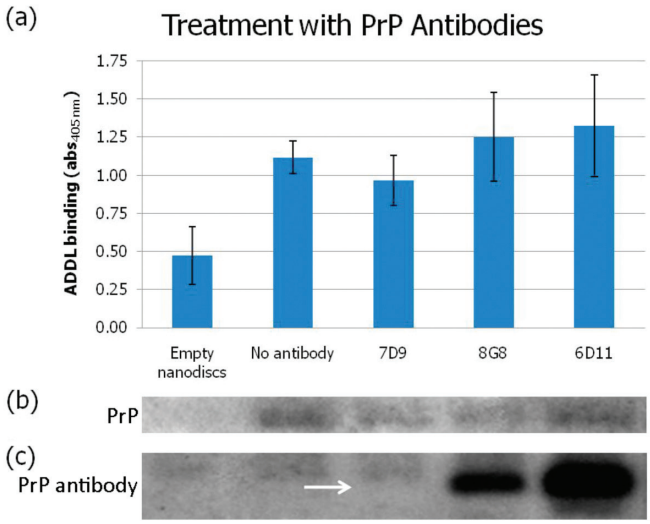

Figure 5. (a) ADDL binding assays show no significant difference in binding after SDS nanodisc treatment with three anti-PrP antibodies (7D9, 8G8, 6D11). (b) Western blot, with lanes corresponding to (a), show that PrP is present in all but empty nanodiscs in relatively similar amounts. (c) The three antibodies differ significantly in their binding affinity for PrP in nanodiscs (white arrow).

Conclusions

The results of this study show promising evidence that nanodiscs can be a useful in vitro platform for studying the interaction of ADDLs with their neuronal receptor. Such a method to study ADDL-receptor interactions would be a major breakthrough in AD research. Utilizing nanodiscs to study ADDL binding at the molecular level should expand the current understanding of how AD begins and lay the foundation for developing new approaches to diagnostics and therapeutics.

Acknowledgments

This research was supported primarily by the Northwestern University Nanoscale Science and Engineering Research Experience for Undergraduates (REU) Program under National Science Foundation (NSF) award number ***EEC – 0755375.*** *Any opinions, findings, conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect those of the NSF.*

References

1 Viola, K. L.; Velasco, P. T.; Klein, W. L. *J. Nutr. Health Aging* **2008,** *12,* 51–57.

2 Klein, W. L.; Lacor, P. N.; De Felice, F.G.; Ferreira, S. T. *Memories: Molecules and Circuits; Springer-Verlag: Berlin Heidelberg,* **2007;** 155–179.

3 National Institute on Aging. *Progress Report on Alzheimer's Disease;* **2008;** 6–7.

4 Lambert, M. P.; Barlow, A. K.; Chromy, B. A.; Edwards, C.; Freed, R.; Liosatos, M.; Morgan, T. E.; Rozovsky, I.; Trommer, B.; Viola, K. L.; Zhang, C.; Finch, C. E.; Krafft, G. A.; Klein, W. L. *Neurobiology* **1998,** *95,* 6448–6453.

5 Klein, W. L. *Neurochem. Int.* **2002,** *41,* 345–352.

6 Krafft, G. A.; Klein, W. L. *Neuropharmacology* **2010,** *59,* 230–242.

7 Lambert, M. P.; Viola, K. L.; Chromy, B. A.; Chang, L.; Morgan, T. E.; Yu, J.; Venton, D. L.; Krafft, G. A.; Finch, C.A.; Klein, W. L. *J. Neurochem.* **2001,** *79,* 595–605.

8 Klein, W. L.; Krafft, G. A.; Finch, C. E. *Trends Neurosci.* **2001,** *24,* 219–224.

9 Bayburt, T. H.; Sligar, S .G. *FEBS Lett.* **2009,** *584,* 1721–1727.

10 Borch, J.; Hamann, T. *Biol. Chem.* **2009,** *390,* 805–814.

11 Nath, A.; Atkins, W. M.; Sligar, S. G. *Biochemistry* **2007,** *46,* 2059–2069.

12 Jones, D. H.; Matus, A. I. *Biochim. Biophys. Acta* **1974,** *356,* 276–287.

13 Leitz, A. J.; Bayburt, T. H.; Barnakov, A. N.; Springer, B. A.; Sligar, S. G. *BioTechniques* **2006,** *40,* 601–612.

14 Laurén, J.; Gimbel, D. A.; Nygaard, H.B.; Gilbert, J. W.; Strittmatter, S. M. *Nature* **2009,** *457,* 1128–1132.

15 Laurén, J.; Gimbel, D. A.; Nygaard, H.B.; Gilbert, J. W.; Strittmatter, S.M. *Nature* **2010,** *466,* E3–E5.