Myelin-mimetic Lipid Multilayers

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ABSTRACT

The myelin sheath is among nature's most fascinating nanoscale assemblies. In spite of its critical role in physiology, several questions on the structure-function relationship of myelin and its components remain unanswered. This is chiefly due to the fact that interactions between the constituents of myelin are particularly difficult to study in intact tissue. The compact, multi- lamellar structure of myelin inhibits or limits access to the interacting membrane surfaces. In addition, characterizing the interaction of purified myelin components has provided only limited information on such things as compositional and environmental effects on myelin structure. Thus, there is a tremendous need to develop novel biomimetic systems that can act as experimental models to help understand this critical component of complex neural systems. Our long-term goal is to create an assembly that closely mimics structural and functional aspects of natural myelin. Here we present data demonstrating the controlled assembly of protein linked multi-bilayers. We also present data supporting the applicability of approaches such as spectroscopic ellipsometry, AFM and fluorescence microscopy to analyze the structural integrity of the assemblies.

Keywords: myelin, biomimetic, lipid, multi-bilayer, protein

1 BACKGROUND

Myelin was mentioned by the physician Virchow in 1854 and clearly defined by Ranvier in 1878 as a sheath that surrounds nerve fibers. This remarkable structure wraps around nerve axons greatly facilitating the conduction of saltatory electrical impulses. Myelin thus generated by a single cell covers a segment of the axon. Portions of the axon are left uncovered at points called the nodes of Ranvier. It is at these nodes that the nervous impulse in the form of an action potential is rejuvenated. The mechanism by which myelin exerts its function is its role as an insulator; preventing the dissipation of ionic gradients. In its formative state, myelin consists of concentric lamellar structures arranged around the axon [1,2]. A cytoplasmic process from the originating glial cell spirals around the axon displacing the previous layer upward and away from the axon. The functional role of the myelin membrane is critically dependent on its unique compositional and structural features, distinguishing it from other plasma membranes. The tight membrane packing has crystal like regularity and at times may contain as many as 225 membrane pairs. Not surprisingly, myelin is comprised of nearly 80% lipid and 20% protein [3], in contrast to most other plasma membranes and naturally occurring biological membrane arrays, such as rod outer segment disk and grana thylakoid membranes, which have nearly equal amounts of lipid and protein.

Given the critical physiological role of myelin, any perturbations to the integrity of this tissue can result in rather drastic consequences. Studies on myelin deficiencies have spanned decades and are described in thousands of papers. These abnormalities span both CNS myelin as well as neuropathies observed in the PNS. Perhaps the most prominent of myelin disorders is Multiple Sclerosis (MS), generally believed to be an auto-immune disorder affecting the CNS. While the significant heterogeneity in the pathology of this condition suggests the possibility of multiple contributing factors, the characteristic of chronic MS is axonal demyelination resulting in reduced nerve conduction velocity [4]. In the PNS, several neuropathies, whether acquired or inherited, are also characterized by significant demyelination. For instance Charcot-Marie-Tooth neuropathy (CMT) type 1 is a chronic demyelinating disease and some subtypes have been mapped to mutations in the PNS myelin protein P_0. Finally, myelin perturbation can also occur as a result of injury, such as that seen in Wallerian degeneration [5] .

1.1 Structure and composition of myelin

Myelin is unique in that it can maintain an orderly structure over the long distances between nodes. Electron microscopic studies show myelin to be a series of alternating dark and light lines, the dense and intraperiod lines respectively. The dense line is formed by the compaction of the cytoplasm and its proteins while each intraperiod line represents apposition of the outer membranes from adjacent layers. X-ray diffraction studies have shown each bimolecular lipid layer to be 5.5 nm thick which alternate with a 3 nm protein (compacted cytoplasm) layer. Further, the separation of membranes is 30-50 Å [6] at the extracellular apposition and slightly reduced at the cytoplasm, probably due to the protrusion of proteins which maintain this spacing.

Myelin once formed is quite stable and it had been suggested that the unique composition of metabolically inert lipid populations may play a role in this enhanced stability [7]. In terms of molar percentages of total lipids in

myelin, the major components are cholesterol, cerebroside, sphingomyelin and glycerophosphatides (ethanolamine, serine and choline). In addition, there are several other classes of lipids which although minor in comparative abundance, are nevertheless important constituents. The lipids in myelin from the PNS are largely the same as that in the CNS, although the relative amounts vary. Comprehensive phylogenetic analyses [8] show that in spite of differences in lipid composition, there are no differential phylogenetic trends in structure, bilayer thickness, membrane separation etc. In spite of the predominance of lipids in myelin composition, proteins play a critical role in myelin structure and function and our long term goal is to exploit their adhesive role in order to create multilamellar assemblies. Several studies have focused on the phosphorylation of the different myelin proteins and their possible role in maintaining myelin integrity and function. With respect to P_0 and MBP, PTMs are postulated to play a significant role in their adhesive function, however the mechanistic role of the modification is still not completely clear. Glycosylation in P_0 is also thought to play a major role in its adhesive function. *The lack of appropriate model systems that can be modified in a systematic fashion has prevented the elucidation of the direct effects of these post-translational modifications in myelin structure and physical properties.*

1.2 Supported lipid bilayers

Supported planar lipid bilayers have been used extensively for various applications and to study molecular interactions [9]. Such systems offer several advantages, not the least of which is control over things such as composition and the facilitation of multiple interrogation tools. For instance, interactions of proteins embedded in a lipid bilayer are well suited for optical spectroscopy [10]. Another aspect that attracts nanotechnologists to the lipid bilayer is the ability to template and assemble supramolecular structures often inspired by biological systems.

Given the above, it is not surprising that efforts have been made to exploit the bilayer platform in myelin studies. However, such efforts have been limited to a few studies. In one study, researchers reconstituted the major dense line by removing detergent from a pre-existing MBP-lipid complex that was isolated from bovine brain [11]. The technique works at re-assembling a multi-bilayer stack from the native components, but lacks control, particularly over the number of bilayers assembled, which limits its usefulness for detailed characterization studies. Basically, it does not provide a model that can be assembled in a controlled fashion with the flexibility to study structure-function, membrane fluidity, and insulating character. Langmuir-Blodgett (LB) techniques have been used by Haas et al. to form multilayers of MBP and negatively charged phospholipid [12]. These structures were analyzed using neutron reflectivity. This paper follows studies by the Riccio group [13,14] on MBP interactions with phospholipids, including the effects of Zn ions [15] which have been reported to have a structuring effect on MBP-lipid interactions [16,17]. LB offers several advantages in model membrane studies, chiefly orientation, which in turn enables good reflectivity curves. However, LB has limitations with some lipids and even in the smallest systems, we have observed that a considerable amount of protein needs to be delivered into the subphase to achieve a reasonable concentration at the lipid surface, notwithstanding constant circulation. Moreover, the kind of real-time AFM and ellipsometry measurements that we have used are not easily facilitated in the LB system. As an alternative, controlled self-assembly appears to be a preferred system for the types of investigations we present here.

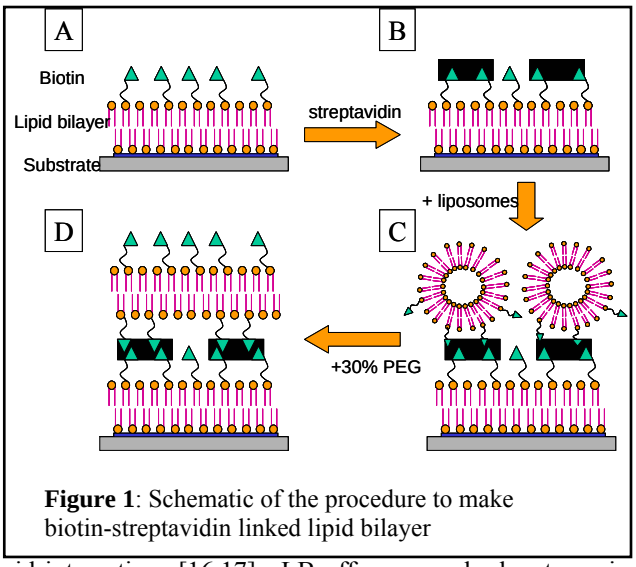

Figure 1: Schematic of the procedure to make biotin-streptavidin linked lipid bilayer

2 MATERIALS

Phosphatidyl ethanolamine (PE), biotinylated PE, fluorescently labeled lipids, vesicle extruders were purchased from Avanti Polar Lipids, Alabaster, AL. Streptavidin and FITC-labeled streptavidin were purchased from Invitrogen, Carlsbad, CA. PEG, coverslips with adhesive coverwells were purchased from Sigma, St. Louis, MO.

3 RESULTS

The results presented here provide support for the long term goals of our project. We demonstrate the ability of controlled self-assembly to generate sequential bilayer stacking using proteins to "stitch" adjacent layers. In addition, we demonstrate the utility of the selected characterization tools to provide information on structure and fluidity of multi-bilayer assemblies.

3.1 Streptavidin linked assembly and AFM

An emphasis in our project is the development of controlled multi-layer assemblies of phospholipids and specific proteins. To create assemblies that will provide a foundation for future integration of myelin components, we

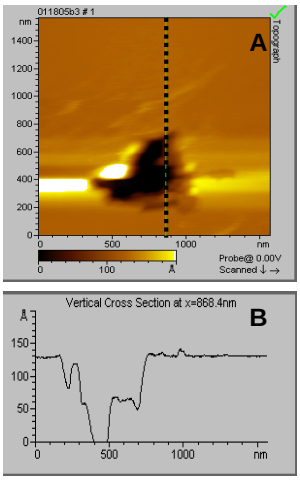

Figure 2. A) mac-AFM of a streptavidin/biotin stacked bilayer assembly formed as shown in Figure 2. **B**) Height plot along the dotted line drawn in **A**.

first developed controlled multi-layer phospholipid assemblies using biotin-streptavidin interactions. In this study, we created a multi-lamellar, three lipid-bilayer assembly held together through biotin/streptavidin conjugation. We first assembled a lipid bilayer, containing a fraction of biotinylated lipids, by vesicle fusion on a planar substrate (Fig 1A). After binding a layer of streptavidin to the surface of the supported membrane, vesicles containing biotinylated lipids were tethered to the surface (1B and 1C). A solution of 30% polyetheylene glycol (PEG) was then added to promote rupture of the vesicles and formation of a second bilayer (1D). Addition of streptavidin followed by biotinylated vesicles and PEG was repeated to form a stack of 3 lipid bilayer membranes, which was characterized by AFM, as shown in Fig. 2A. The AFM image shows that the assembly is relatively smooth, with a roughness of < 0.5 Å. We then used the AFM tip to puncture a hole in the protein/lipid assembly in order to observe the underlying layers. The height graph shown in 2B corresponds to the line drawn in 2A. This graph clearly shows that this assembly is in fact constructed from multiple bilayers as indicated by the step in Figure 2B. This step is ~70 Å, which is consistent with the expected height of a single lipid bilayer plus a streptavidin intermediate layer. The lower layers are not observable because the tip does not have enough freedom to translate along the z-direction. These results were corroborated by neutron reflectivity measurements performed at LANSCE (data not shown). Overall, these results indicate that using self-assembly techniques with proteins to mediate lipid bilayer attachment is indeed a viable approach, which we can build upon to stack several bilayers akin to a myelin structure.

3.2 Measurement of fluidity in a stacked assembly

Upon establishing a procedure for consistent assembly of streptavidin- linked multilayers, we performed Fluorescence Recovery After Photobleaching (FRAP) using an inverted fluorescence microscope to measure fluidity of specific bilayers within the assembly. In this case, the bilayers were made with DPPE liposomes with varying

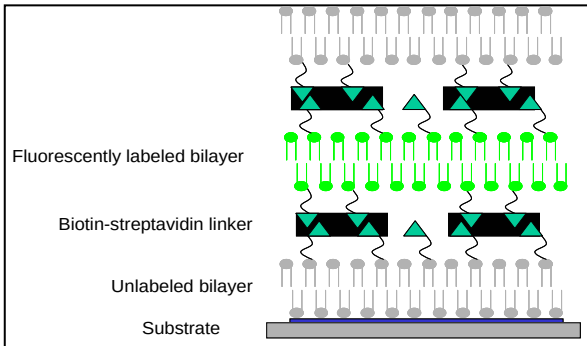

Figure 3: Schematic of assembly used for fluidity measurement experiments

amounts of biotinylated-PE incorporated. When desired, Texas Red labeled PE was incorporated for a specific bilayer. (schematic in Fig 3). The multilayer assembly was formed as described above. FRAP measurements were conducted immediately after assembly. Briefly, a spot was exposed to intense fluorescence and bleached. Recovery of fluorescence due to lateral mobility of lipids was observed over 25 minutes. As seen in Table 1 panel A, as the concentration of biotinylated lipids was increased, the fluidity was reduced, most likely due to enhanced adhesion.

Biotin Conc.	Layers	Diffusion Coeff. (μ^2/sec)
Panel A		
0%	TxRed	4.1 ± 0.1
0.2%	TxRed	3.5 ± 0.1
2%	TxRed	2.6 ± 0.1
20%	TxRed	1.33 ± 0.01
Panel B		
0.2%	TxRed	3.5 ± 0.1
	TxRed + SA	3.60 ± 0.02
	TxRed + SA + unlabeled	3.8 ± 0.1
	Unlabeled + SA + TxRed	1.9 ± 0.1

Table1: FRAP - lateral mobility

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Panel B shows the effects of bilayer position on fluidity. Here, biotinylated PE was maintained at 0.2 mole % of total lipid and a single bilayer was first formed, followed by addition of streptavidin and the second bilayer. Interestingly, we did not see a reduction in fluidity as we expected. However, when the Texas Red bilayer was assembled over a streptavidin and unlabeled bilayer, the fluidity was reduced. Taken together, these results suggest that fluidity may be altered as a result of position within the multilayer assembly and the extent of "protein-mediated stitching". A more detailed examination of fluidity with complex multilayers will provide a better insight into this process.

3.3 Spectroscopic ellipsometry of assembled bilayers

An initial substrate-supported phospholipid bilayer containing biotinylated lipids was exposed to streptavidin and then incubated with biotinylated-lipid containing vesicles. Spectroscopic ellipsometry of lipid membranes in aqueous conditions are now a routine practice and are able to measure changes as small as 1-5 Å in layer thickness [18]. Careful structural characterization of the lipid assemblies indicate that lipid bilayers with a uniform thickness were formed. Further, when we added streptavidin, we observed a time dependent increase in thickness, ultimately leveling out at about 38Å after 5 hours (Fig 4).

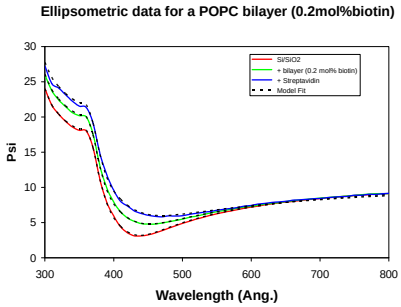

Figure 4. Spectroscopic ellipsometry measurement of streptavidin addition to a lipid (POPC) bilayer containing 0.2% biotinylated lipid. Modeling of the spectroscopic data gave the thickness of SiO_2 = 6.6 nm, bilayer = 4.7 nm and streptavidin layer = 2.7 nm. Taking a close-packed streptavidin layer to be ≈5 nm thick, streptavidin covers ≈54% of the bilayer.

4 CONCLUSIONS

In this paper, we demonstrate 1) that formation of stacked bilayer assemblies using protein mediated assembly is a viable approach, 2) that this approach facilitates the application of bilayer characterization methods without major alterations to the experimental set-up, and 3) that measurements made correlate with projected outcomes. We believe this first attempt at using self-assembly to assemble protein mediated lipid multi-bilayers will set the stage for our future experiments aimed at mimicking myelin-like structures more closely.

5 ACKNOWLEDGMENTS

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