A study of lipid recognition and membrane binding by the human oxysterol-binding protein (OSBP)

by

Parthajit Mukherjee, B.Sc.

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to the

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“In dedication to *Maa, Baba* and *Aadi*  

– the three pillars of my life”
Abstract

Recent studies have established oxysterol-binding protein (OSBP) and members of the OSBP-related protein (ORP) family as global cellular sterol sensors that participate in non-vesicular anterograde transport of monomeric sterols from the endoplasmic reticulum to other organelles such as the Golgi and the plasma membrane. By exchanging sterols for phosphoinositides, these multi-domain proteins change the bilayer composition at membrane contact sites and thus, regulate various signaling pathways.

Despite the wealth of knowledge garnered from the study of fluorescent/radiolabeled ligand-protein interactions and inter-vesicular lipid transfer assays in vitro, the precise nature of the association of ORPs with organellar membranes and the factors modulating such interactions have remained largely enigmatic. The goal of my project was to characterize the behaviour of human OSBP using a label-free analytical technique called dual polarization interferometry (DPI). This technique enables surface-immobilization of phospholipid vesicles to observe and analyze the behaviour of proteins towards adsorbed bilayers.

From my investigation, I found that OSBP prefers binding to membranes containing anionic phospholipids, such as phosphatidylinositol-4-phosphate (PI(4)P), over membranes made up of neutral phosphatidylcholine.
In the presence of PI(4)P, the wild-type protein clearly demonstrated a rapid bilayer association, followed by PI(4)P extraction and a slower dissociation, in a dosage-dependent fashion. The OSBP-related domain (ORD) mutant, OSBP-HH/AA, due to its impaired ability to extract PI(4)P, failed to dissociate from the membrane while the pleckstrin homology domain (PHD) mutant, OSBP-RR/EE, could not associate with membranes at all. The presence of sterols did not alter OSBP’s affinity for PC membranes despite a two-fold increase in protein adsorption per unit area in the presence of cholesterol in the membrane, compared to 25-hydroxycholesterol.

Both cholesterol and 25-hydroxycholesterol competed with 22-NBD-cholesterol for the binding site in the ORD of OSBP, with resulting $EC_{50}$ values of $15.6 \pm 0.7$ nM for the former and $5.0 \pm 0.5$ nM for the latter. OSBP also transferred ORD-bound fluorescent cholesterol to acceptor vesicles, but the rate remained unaltered upon incorporation of PI(4)P in those membranes.

These results provide useful insight into the preferential association of OSBP with membranes containing specific recognizable ligands, such as sterols and PI(4)P, and help build a molecular level description of the mechanism of this protein.
Acknowledgments

This work would not have been a reality without the invaluable support of several great individuals to whom I feel forever indebted.

First and foremost, I express my heartfelt gratitude for my supervisor, Dr. Jeffrey Atkinson, who has been a role model over the years. His undeterred faith in my ability had not only encouraged me to stay focused on this project but also helped me overcome the challenges that came along every step of my work. Under his keen mentorship, I learned to appreciate the scientific approach of solving problems and develop a habit of critical thinking. His wise academic advice and professional assistance have made my journey at Brock a successful and memorable experience.

The unparalleled enthusiasm of my supervisory committee members, Drs. Heather Gordon and Alan Castle, has been instrumental in nourishing my interpersonal skills. To say that I'm thankful for their continual guidance and insightful critiques of my work will be a mere understatement.

I sincerely thank our collaborator Dr. Neale Ridgway and his group from Dalhousie University (Halifax, Canada) for the generous gift of the baculovirus constructs for protein expression and invaluable help during the crucial times of my research.

I wave to my colleagues from the Atkinson lab, Drs. Matilda Baptist and Candace Panagabko, in appreciation of their priceless friendship and constant support throughout the duration of my stay at Brock. I also express my heartfelt thanks to Sina Mazinani and Hasam Madarati for believing in my potential and motivating me to push forth. Many thanks to Dr. Mark Lukewich and Julie Domitrek for being such a blissful company, both in the
school and away. I value their friendship and respect them for providing me a great environment for teaching and learning.

I would also like to extend my wholehearted appreciation for the scientific expertise of Dr. Marcus Swann from Biolin Scientific (Stockholm, Sweden) that enabled smooth operation of the DPI instrument, technical troubleshooting, and data analysis.

This achievement would not have been possible without my parents. I feel blessed to have them by my side through every high and low tides of life.

Words cannot explain the contributions of my fiancée, Aadi. Her presence, and ceaseless love and care, made this journey look far less challenging. I thank her from the bottom of my heart for bringing out the best in me.
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<td>7αHC</td>
<td>7-α-hydroxycholesterol</td>
</tr>
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<td>7α,25DHC</td>
<td>7-α,25-dihydroxycholesterol</td>
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<td>7βHC</td>
<td>7-β-hydroxycholesterol</td>
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<td>7-ketocholesterol</td>
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<td>7OOHC</td>
<td>7-α/β-hydroperoxycholesterol</td>
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<td>27-hydroxycholesterol</td>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<td>ACAT</td>
<td>acetyl-CoA-cholesterol acetyltransferase</td>
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<tr>
<td>AcMNPV</td>
<td><em>Autographa californica</em> multicapsid nucleopolyhedrovirus</td>
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<tr>
<td>ALPS</td>
<td>amphipathic lipid packing sensor</td>
</tr>
<tr>
<td>ANK</td>
<td>ankyrin</td>
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<td>APS</td>
<td>ammonium persulfate</td>
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<td>BEVS</td>
<td>baculovirus expression vector system</td>
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<td>BHK</td>
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<td>CTL</td>
<td>cholestatrienol</td>
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<td>Acronym</td>
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<tr>
<td>CYP</td>
<td>cytochrome P-450</td>
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<td>D</td>
<td>dual adaptor of phosphotyrosine and 3-phosphoinositides 1</td>
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<td>DHE</td>
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<td>E</td>
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<td>EDTA</td>
<td>endoplasmic reticulum/reticulum</td>
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<td>ERCs</td>
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<td>F</td>
<td>four-phosphate-adaptor protein 2</td>
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<td>FAPP2</td>
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<tr>
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<tr>
<td>FFAT</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>FRAP</td>
<td>Förster resonance energy transfer</td>
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<td>G</td>
<td>glycolipid transfer protein</td>
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<td>GLTP</td>
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<td>IgM</td>
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<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
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<td>L</td>
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<tr>
<td>LCAT</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LDL</td>
<td>lipid transport protein</td>
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<td>LTP</td>
<td>lipid transport protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
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<tr>
<td>LXR</td>
<td>liver X receptor</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MCS</td>
<td>membrane contact site</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>MNPV</td>
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<tr>
<td>MSP</td>
<td>major sperm protein</td>
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<td>mTOR</td>
<td>mechanistic target of rapamycin</td>
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<td>nucleopolyhedrovirus</td>
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<tr>
<td>OB</td>
<td>occlusion body</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>ODV</td>
<td>occluded/occlusion-derived virus</td>
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<tr>
<td>ORD</td>
<td>OSBP-related domain</td>
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<td>OSBP-related protein</td>
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<tr>
<td>Osh</td>
<td>OSBP homolog</td>
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<td>OSBP</td>
<td>oxysterol-binding protein</td>
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<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PC</td>
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<td>PITP</td>
<td>phosphatidylinositol transfer protein</td>
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<td>plasma membranes</td>
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<td>PS</td>
<td>phosphatidylserine</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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</table>
R
ROS reactive oxygen species

S
SCAP SREBP-cleavage-activating protein
SCP2 sterol carrier protein 2
SDS sodium dodecyl sulfate
Sf *Spodoptera frugiperda*
SLB supported lipid bilayer
SNPV single nucleopolyhedroviruses
SPR surface plasmon resonance
SREBP s sterol regulatory element binding protein
StAR steroidogenic acute regulatory protein
START StAR-related lipid transfer
SULT2B1b/2A1 hydroxysteroid sulfotransferase 2B1b/2A1

T
TE transverse electric
tEMED tetramethylethylenediamine
tGN trans-Golgi network
tM transverse magnetic
tMB 3,3′,5,5′-tetramethylbenzidine
tis tris(hydroxymethyl)aminomethane

U
UDP uridine diphosphate
UGT UDP-glucuronosyltransferase

V
VAP-A VAMP-associated protein A
VAMP vesicle-associated membrane protein

W
wt wild-type
Chapter 1

INTRODUCTION

Oxysterol-binding protein (OSBP) is a sterol transfer protein known to facilitate anterograde transport of cholesterol and its oxygenated derivatives from the endoplasmic reticulum to other organellar membranes in eukaryotic cells. This chapter provides an overview of the origin and significance of oxysterols along with a description of various non-vesicular modes of intracellular lipid trafficking. Also highlighted are the biological roles of the members of the OSBP-related protein (ORP) family in diverse signaling pathways and the molecular interactions of their structural domains with a wide range of lipid ligands. The current progress in the field of ORP research and the popular models explaining the mechanism of ligand exchange by mammalian OSBP and yeast Osh4 are discussed here. A breakdown of the primary goal of this project is provided with detailed background information on the techniques utilized to achieve the research objectives.
1.1. Oxysterols

Oxysterols are oxygenated derivatives of cholesterol that have been extensively researched for their involvement in sterol homeostasis. In recent years, these biologically active compounds have gained much interest due to their newfound roles as regulators of signaling pathways, as ligands for novel receptors, as components of the cell’s immune response and as crucial biomarkers for atherosclerosis and neurodegenerative diseases. This section will, however, focus on the origin and role of these chemical species in the regulation of cholesterol metabolism.

1.1.1. Origin of oxysterols

Oxysterols can be classified into two major varieties based on the number of structural modifications. While primary oxysterols have single substitutions either in the side chain or the aromatic rings of cholesterol, the secondary oxysterols feature multiple functionalities including hydroxyl, epoxy, keto, or hydroperoxy groups (Olkkonen and Lehto, 2004). Oxysterols are known to be synthesized both enzymatically as well as non-enzymatically and can either be produced endogenously or acquired from the diet. Figure 1 provides a summary of the chemical structures of various oxysterols originating either directly or indirectly from cholesterol. In the chemical structure of cholesterol, the presence of a double bond between C5 and C6 positions makes the seventh carbon of the B ring prone to any free-radical attack, thereby causing non-enzymatic transformation of the parent molecule. 7-α/β-hydroperoxysterol (7OOHC) is produced in this fashion (Brown and Jessup, 2009). The biological function of this species has remained largely elusive despite its presence in low-density lipoproteins (LDL) (Brown et al., 1997; Terao, 2014).
Figure 1: Chemical structures of some oxysterols. Oxysterols originate from cholesterol by the action of various cytochrome P450 enzymes (CYPs) or from reactive oxygen species (ROS). Some members, such as 24(S),25-epoxycholesterol, originate from the cholesterol precursor by the action of CYP46A1.

While the non-enzymatic oxidation of cholesterol via free radicals remained the centre of research during the 80s and the 90s, the post-2000 era
witnessed a shift of interest to enzymatically-derived oxysterols. Most of these enzymes belong to the cytochrome P450 (CYP) superfamily located in the ER. For example, 22(R)-hydroxycholesterol (22HC) is produced by CYP11A1 as an intermediate during the biosynthesis of steroid hormones (Mast et al., 2011). Likewise, the formation of 27-hydroxycholesterol (27HC) is catalyzed by CYP27A1 in several cell types, such as macrophages, during the “acid pathway” of bile acid biogenesis (Cali and Russell, 1991; Björkhem et al., 2002). Some oxysterols are generated to serve unique purposes in specialized cells, such as 24(S)-hydroxycholesterol (24HC) by CYP46A1 in neurons (Bretillon et al., 2007) and 7α,25-dihydroxycholesterol (7α,25DHC) by CH25H (cholesterol 25-hydroxylase – a reductase/hydroxylase located in the mitochondrial matrix) and CYP7B1 in B-lymphocytes and dendritic cells (Cyster et al., 2014). Some oxysterols are also generated by the action of multiple enzymes. A common example is 24(S),25-epoxycholesterol (24,25EC), which is not derived directly from cholesterol. Instead, it is produced by CYP46A1 from desmosterol, a penultimate member of the classical cholesterol biosynthetic pathway, via a shunt of the pathway through a dioxidosqualene intermediate (Ali et al., 2013; Wang et al., 2014).

Several oxysterol species can arise via both enzymatic and non-enzymatic reactions. A key example is 7-ketocholesterol (7KC). It is a common breakdown product of 7OOHC but is also known to be converted from 7-dehydrocholesterol, an alternate product of lanosterol in the cholesterol biogenesis (the primary product being desmosterol), by CYP7A1 (Shinkyo et al., 2011). Other non-enzymatic degradation products of 7OOHC include 7α-hydroxycholesterol (7αHC) and 7β-hydroxycholesterol (7βHC). Nevertheless, they are also generated by CYP7A1 and the cortisone reductase 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), respectively (Russell, 2003; Mitić et al., 2013). 7αHC is a major intermediate in the “neutral pathway” of bile acid biogenesis. The most commonly studied species of
oxysterol, 25-hydroxycholesterol (25HC), is an inhibitor of cholesterol biosynthesis and LDL receptor expression in cell models. A remarkable uniqueness of 25HC stems from its diverse modes of origin. It is the only oxysterol that is generated by three independent routes: autoxidation of aged or crystalline cholesterol (Diczfalusy, 2013), as a major product of CH25H activity (Lund et al., 1998) and also in minor quantity by CYP27A1, CYP46A1 or CYP3A4 (Luu et al., 2016). However, due to its low abundance in human tissues compared to other non-enzymatically produced oxysterols, autoxidation of 25HC \textit{in vitro} and \textit{in vivo} remains questionable at present.

\subsection*{1.1.2. Oxysterol metabolism}

Oxysterols primarily undergo esterification of the $\beta$-hydroxyl group at the C3 position by two classes of acetyltransferases which are also known to act on cholesterol: lecithin-cholesterol acetyltransferase (LCAT) (Glomset, 1962) and acetyl-CoA-cholesterol acetyltransferase (ACAT) (Chang et al., 1993). In addition to the formation of various oxysterol esters, hydroxylation of 25HC by CYP7B1 gives rise to 7α,25DHC, as mentioned earlier (Martin et al., 2001). Sulfotransferases, such as hydroxysteroid sulfotransferase 2B1b and 2A1 (SULT2B1b and SULT2A1), also modify oxysterols by sulfonating either the 3β-hydroxyl or both 3β- and 25β-hydroxyl groups of 25HC (Li et al., 2007; Ren et al., 2014). Sulfonation has been reported to alter the native function of various oxysterols and an increased level of circulating oxysterol sulfates leads to several pathological conditions (Sánchez-Guijo et al., 2015). Like sulfonation, oxysterols can also undergo glucuronide conjugation. This is evident from the high levels of sulfate/glucuronide double conjugates of 24HC and 27HC in the serum and urine of children with severe cholestatic liver disease (Meng et al., 1997). Enzymes responsible for the glucuronidation of oxysterols belong to the uridine diphosphate (UDP)-glucuronosyltransferase
(UGT) family that account for the majority of phase II metabolism in humans (Björkhem et al., 2001; Verreault et al., 2006).

1.1.3. Impact of oxysterols on cholesterol homeostasis

Kandutsch and co-workers formulated the “Oxysterol Hypothesis of Cholesterol Homeostasis” in 1978 when they postulated that cholesterol exhibits a negative regulatory effect on its own biosynthesis, not by itself, but with the help of its oxygenated forms, called oxysterols. Using mouse lymphocytes (Chen et al., 1975), human fibroblasts (Breslow et al., 1975) and Chinese hamster lung cells (Chen et al., 1979), they were able to show that an impure sample of cholesterol containing trace amounts of oxysterols, was able to inhibit cholesterol synthesis whereas pure cholesterol did not (Kandutsch et al., 1978). Although their work was initially subjected to scrutiny and criticism, almost four decades of research has reshaped the historical hypothesis and established beyond doubt that oxysterols play a major role in the regulation of cholesterol homeostasis at both transcriptional and post-translational levels.

1.1.3.1. Transcriptional regulation:

Two major transcriptional regulators of cholesterol metabolism are the sterol regulatory element binding proteins (SREBPs) and the liver X receptors (LXRs). An example of a gene transcribed by the former is 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase and an example of a gene regulated by the latter is LDL receptor (Gill et al., 2008).

Specific isoforms of SREBPs control the expression of genes involved in both cholesterol biosynthesis and uptake. Mechanistically, SREBPs occur in
the ER as membrane-bound precursors and they are associated with a cholesterol-sensing SREBP-cleavage-activating protein (SCAP) which undergoes conformational changes upon ligand binding. In response to low cellular cholesterol levels, SCAP facilitates localization of the SCAP-SREBP complex to the Golgi where SREBP is activated by sequential proteolytic cleavage and released to the nucleus as a soluble transcription factor to target specific genes involved in cholesterol synthesis (Goldstein et al., 2006). When there are sufficient levels of cholesterol in the cell, cholesterol binds to SCAP which prevents the departure of SCAP-SREBP complex from the ER by a retention protein, named Insig. This, in turn, prevents SREBP activation (Brown et al., 2002).

Due to the presence of a substantial amount of evidence in support of the fact that only cholesterol, and not oxysterol, can bind to SCAP (Adams et al., 2004), it is not surprising to question the validity of the Oxysterol Hypothesis of Cholesterol Homeostasis. However, the discovery that Insig acts as an oxysterol sensing protein at the ER has certainly turned the tide in favor of the hypothesis (Radhakrishnan et al., 2007). Numerous studies have supported the reciprocity of the sterol-binding properties of SCAP and Insig. While SCAP can bind to the tetracyclic steroid framework and the 3β-hydroxyl group of cholesterol, the presence of a polar group on the side-chain disrupts this association. In contrast, binding to Insig is made possible only by a polar group on the side-chain. Therefore, a revised model supports that both cholesterol and oxysterol regulate cholesterol homeostasis via two very similar, yet separate, modes of inhibiting SREBP activation: binding of cholesterol to SCAP and binding of oxysterol to Insig.

Unlike SREBPs, regulation of cholesterol homeostasis via LXRs is a consequence of direct binding of oxysterols with this superfamily of ligand-activated nuclear hormone receptors. LXRs are incapable of binding to cholesterol and cholesterol esters but can effectively bind oxysterols because
of their structural preference to accommodate side-chain hydroxyl, keto or oxopxide groups (Janowski et al., 1999). The levels of the oxidized derivatives closely reflect the cellular cholesterol status. Upon ligand binding, LXR
s dimerize with retinoid X receptors and recruit co-activators that result in transcriptional activation of a network of genes involved in reverse cholesterol transport, cholesterol catabolism and lipogenesis (Willy and Mangelsdorf, 1997; Ignatova et al., 2013). In cells and tissues experiencing high cholesterol flux, LXR
s upregulate the expression of ATP-binding cassettes (ABC). ABCA1 and ABCG1 are two such proteins which play an important role in cholesterol removal from macrophages (Vaughan and Oram, 2006). Various in vitro and in vivo experiments have suggested that naturally occurring oxysterols with oxygenated side-chains, such as 24-, 25- and 27HCs, serve as natural ligands for LXR
s (Brown and Jessup, 2009). Overexpression and gene knockout studies have provided compelling evidence in favor of the bona fide involvement of endogenous 24,25EC in transcriptional control of ABCA1 in an LXR-dependent manner (Wong et al., 2008).

1.1.3.2. Post-translational regulation:

Oxysterols are involved in fine-tuning the activity of various enzymes. HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonate – a classic rate-limiting step of cholesterol biogenesis, is known to be degraded by side-chain-derivatized oxysterols via Insig signaling (DeBose-Boyd, 2008). Another example is inhibition of 24-dehydrocholesterol reductase by 24,25EC (Zerenturk et al., 2012). Certain ring-derivatized oxysterols (such as 7αHC) are known to promote degradation of squalene monooxygenase, another rate-limiting enzyme of cholesterol synthesis pathway (Gill et al., 2011).
1.2. Intracellular Lipid Trafficking

Biogenesis and intracellular distribution of lipids are highly compartmentalized phenomena. Although different lipids are known to originate from various organelles, the primary site of lipogenesis in eukaryotic cells is the endoplasmic reticulum (ER). Unlike proteins, lipids lack specific targeting motifs to mediate their cellular distribution, yet over a thousand chemically distinct species of lipids in eukaryotic cells find their niche in specific compartments giving rise to characteristic compositions of each organelle membrane. For example, plasma membrane (PM) in cultured fibroblasts is known to harbour ~60 – 80 % of total cellular cholesterol on a molar basis, which represents 30 – 40 % of the PM surface (Lange et al., 1989; Lange, 1991; Liscum and Munn, 1999). In spite of being synthesized at the ER, cholesterol occupies only 3 – 6 % of ER membrane, which is a mere 0.5 – 1 % of the total cholesterol content of a cell (Lange et al., 1999; Liscum and Munn, 1999; Maxfield and Wüstner, 2002). The interior of the cell can therefore be envisioned as a complex network of highways by means of which diversified cargoes of lipid are transported in every direction.

1.2.1. Vesicle-mediated transport

The intracellular transport of lipids occurs via different mechanisms. Among these, the most prominent means is the vesicle-mediated transport. It is an active phenomenon wherein a portion of a donor membrane forms tubular structures that encapsulate a payload of specific proteins and lipids, bud-off and subsequently fuse with an acceptor membrane (Gillon et al., 2012). Since lipids form the basic structural backbone of transport vesicles, it is apparent that large amounts of lipids are transferred between membranes by endocytic and exocytic pathways. Specialized signaling motifs in the
cytoplasmic domains of transmembrane proteins help them interact with other peptides which facilitates their inclusion in budding vesicles.

More sophisticated cargo sorting can occur, as is the case for LDL receptors internalized by coated pits (Goldstein et al., 1981). Upon delivery to sorting endosomes, LDL is dissociated from its receptor due to the acidic pH of the endosomal compartment (Maxfield and McGraw, 2004). The nascent vesicles budding from the surface of endosomes have a high surface area to volume ratio compared to that of the organelle itself. This results in efficient sorting and removal of LDL receptors into the vesicles, while the soluble LDL is retained.

Unlike proteins, the underlying mechanisms of lipid sorting into transport vesicles are not well understood. In the PM, cholesterol associates with sphingolipids to form lipid-ordered micro-domains. Experimental evidence suggests that spatial segregation of protein contents of synaptic vesicles into lipid raft domains is facilitated by sterols (Lv et al., 2008). Using a lipidomics approach, Simons and co-workers demonstrated that the trans-Golgi network (TGN) of *S. cerevisiae* was capable of sorting membrane lipids, such as ergosterol, into secretory vesicles that exhibited higher membrane order (Klemm et al., 2009). However, the vesicle-mediated transport may not be the major mode of sterol trafficking inside a cell. For instance, only 20% reduction in the transport of newly synthesized cholesterol from the ER has been observed in baby hamster kidney (BHK) fibroblasts treated with vesicular transport inhibitor Brefeldin A (Damke et al., 1991; Heino et al., 2000). Likewise, ergosterol transport remains unaffected in yeast cells expressing a defective version of Sec18, an essential protein for anterograde vesicle transport from the ER (Baumann et al., 2005).

Maxfield’s laboratory has studied the intracellular sterol transport using a naturally occurring fluorescent sterol named dehydroergosterol
(DHE). By measuring the fluorescence recovery after photobleaching (FRAP), his group was able to estimate the rate of transport of DHE into endocytic recycling compartments (ERCs) in Chinese hamster ovary (CHO) cells. According to them, the magnitude of fluorescence recovery was reduced only slightly when ATP was depleted from the cells (Hao et al., 2002). In light of the fact that ATP provides energy that drives vesicular transport, these results suggest that a significant proportion of sterol cargo in the cell is delivered to its destination by non-vesicular means.

1.2.2. Non-vesicular transport

Non-vesicular movement of lipids involve a multitude of closely-related processes as outlined in Figure 2. These processes can be broadly classified into three modes: spontaneous, transbilayer and lipid transfer protein (LTP)-mediated transport.

**Figure 2: Various modes of non-vesicular lipid transport.** Monomeric lipids can be transported in a non-vesicular fashion inside the cell by three mechanisms: spontaneous, transbilayer (flip-flop or lateral diffusion), and LTP-mediated transport. LTP-mediated transport can be unidirectional (from donor membrane to acceptor membrane) or bidirectional (shuttle/exchange between two membranes). Flip-flops can be spontaneous or mediated by flippases and translocases (Lev, 2010).
1.2.2.1. Spontaneous transport:

Spontaneous disruption of lipid-lipid interaction at the membrane surface, followed by desorption of lipid molecule in a cavity formed in the aqueous phase, allow for free diffusion of lipids in their monomeric forms through the cytosol, before being incorporated into a bilayer. Lipid movement/exchange can take place between two separate membrane bilayers or within the lateral plane of the same bilayer (Lev, 2010). Sometimes, two membranes collide and result in lipid exchange. A slightly modified form of collision-mediated transfer involves extension of the lipid from the donor membrane surface, which not only reduces the energy cost of the transfer to the acceptor membrane but also increases its probability. This is called activated collision. While aqueous diffusions mainly occur at low membrane concentrations, collision-based transfers take place at high membrane concentrations. Both processes are concentration-dependent. On one hand, aqueous diffusion follows first-order reaction kinetics in which the rate is defined by concentration of the donor membrane only (Lev, 2010). On the other hand, collisions are second-order processes wherein the transfer rates are determined by the concentrations of both donor and acceptor membranes (Lev, 2010).

Spontaneous diffusions are energetically unfavorable and extremely slow processes in the aqueous milieu of the cytosol due to the hydrophobic nature of most lipids. The rates of such transfers are proportional to the aqueous-phase solubility of the participating sterol (or phospholipid) species. For example, $t_{1/2}$ of spontaneous transfer of 25HC, cholesterol and cholesterol oleate have been reported as 2 min, 2 h and $10^7$ h, respectively (Lev, 2010). Therefore, for this process to be more efficient, the donor and recipient membranes should be in close proximity. Existence of small cytosolic gaps, measuring 10-30 nm, formed by close apposition of ER membranes with other organelles, such as Golgi, PM, peroxisomes, mitochondria, etc., has attracted
a lot of interest in recent years. Current studies have elucidated the
significance of these membrane contact sites (MCSs) in lipid biosynthesis,
Ca\textsuperscript{2+} signaling and transport of metabolites (Helle et al., 2013; Phillips and
Voeltz, 2015). MCSs not only facilitate non-vesicular transport of lipids by
spontaneous diffusion but they also provide an active platform for lipid
transport proteins (LTPs) to function (Mesmin et al., 2013).

1.2.2.2. Transbilayer transport:

Lipids can be transported between the two leaflets of a membrane
bilayer either spontaneously or by the help of flippases and translocases
(Hankins et al., 2015). Although the flip-flop mechanism of transbilayer lipid
exchange does not directly contribute to inter-organellar lipid flux, it is
thought to signal vesicle fission and fusion by presenting specific lipids on the
surface. In addition, by changing the composition of bilayer leaflets, flip-flop
transition enables creation of lipid micro-domains (or lipid rafts) within the
bilayer and influences membrane curvature depending on the intracellular
need (Contreras et al., 2010). While most translocases utilize the energy of
ATP hydrolysis to irreversibly transport phospholipids across the leaflets,
some flippases, called scramblases, are energy-independent and engage in
establishing a reversible and bidirectional equilibrium of phospholipids.

1.2.2.3. Transport mediated by lipid transfer proteins (LTPs):

LTPs are capable of recognizing donor surfaces, either selectively via
specialized structural motifs or non-selectively by virtue of the dual
functionality of their lipid-binding domains to sense lipid gradients. Based on
the properties of their hydrophobic binding pockets, LTPs extract and
transfer selective lipid monomers by shielding them from the aqueous environment and delivering them to their destined locations. The earliest account of study in this area dates back to 1968, when a non-dialyzable, heat-labile, and trypsin-sensitive cytosolic factor, capable of transferring phosphatidylcholine (PC) between microsomes and mitochondria in rat hepatocytes, was first isolated by Zilversmit and colleagues (Wirtz and Zilversmit, 1968). These factors were later identified as proteins and were accordingly named “phospholipid transfer proteins” (Wirtz and Zilversmit, 1970). Since this discovery, many LTPs have been isolated and characterized in bacteria, eukaryotes and plants.

Although extensive research has been done over the past 30 – 35 years, the precise modes of action of LTPs have remained somewhat enigmatic. Moreover, the fact that these carriers mediate lipid transport against the concentration gradient over localized membrane microenvironment paints a rather intriguing picture in which their lipid sensing/transfer activity serves a secondary function in the regulation of complex metabolic and signaling pathways. Today, these proteins are subdivided into various families based on their structural and functional similarity, including but not limited to phosphatidylinositol (PI) transfer protein (PITP), steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain proteins, glycolipid transfer protein (GLTP), sterol carrier protein 2 (SCP2), ceramide transfer protein (CERT) and oxysterol-binding protein (OSBP)-related proteins (ORPs).

For the sake of simplicity, the information provided below is limited to ORPs only. Readers interested in other LTPs can find extensive reviews elsewhere (Lev, 2010; Clark, 2012; McDermott and Mousley, 2016).
1.3. Oxysterol-Binding Protein (OSBP)-Related Proteins (ORPs)

Oxysterol-binding protein (OSBP)-related proteins (ORPs), constitute a large eukaryotic gene family that includes both mammalian proteins as well as those encoded by the OSBP homologue (OSH) gene family in yeast (Olkkonen et al., 2006). They primarily act as intracellular transporters of sterols and other phospholipids between various membranes compartments, such as ER, Golgi and PM. Since their discovery by Taylor and Kandutsch in 1980s as cytoplasmic factors capable of binding oxygenated derivatives of cholesterol and suppressing HMG-CoA reductase (Taylor et al., 1984; Taylor and Kandutsch, 1985), a significant amount of information about the nature and mode of action of ORPs has been acquired by researchers.

Interestingly, the hypothesis that OSBP was involved in 25HC-mediated suppression of sterol-regulated genes (Brown and Goldstein, 1974; Kandutsch and Chen, 1974; Lagace et al., 1997), was discarded when 25HC-induced inhibition of HMG-CoA reductase was found to be independent of OSBP (Nishimura et al., 2005). Some aspects of the precise role of ORPs in intracellular trafficking of lipids, especially phosphatidylinositol phosphates (PIPs), still remain unknown.

Recent advances in the field of ORP research have revealed that these proteins localize at MCSs and possess the ability to simultaneously associate with the two closely-apposed membranes and facilitate lipid transport between them (Mesmin et al., 2013). Therefore, in order to understand the mechanism of action of these LTPs, it is crucial to have a clear comprehension of their structural characteristics.
1.3.1. Domain organization of ORPs

The yeast, *Saccharomyces cerevisiae*, has seven Osh proteins while 12 ORPs have been identified in human with four additional splice variants (Lehto *et al.*, 2001). The organization of domains in both human and yeast is shown in Figure 3A. The founding member the ORP family, namely OSBP, is characterized by a C-terminal sterol-binding/transfer domain (Ridgway *et al.*, 1992; Lehto *et al.*, 2001), commonly known as OSBP-related domain (ORD) and an N-terminal pleckstrin homology domain (PHD) that recognizes and targets phosphoinositides with high specificity (Lemmon and Ferguson, 2001), facilitating protein localization to PIP-enriched membranes of organelles such as the Golgi (Levine and Munro, 2002) and PM (Lehto *et al.*, 2005). The ORD is found in all members of this family and is marked by a highly-conserved signature motif EQVSHHPP near its N-terminus. Some proteins of the Osh family, in fact, possess only the ORD. The examples are Osh4, -5, -6 and -7. Based on the gene structure and amino acid homology, human ORPs are classified into six subfamilies (Lehto *et al.*, 2001). The members are designated as long (L) and short (S) variants based on the presence of the N-terminal PHD in addition to the ORD.

The yeast family is subdivided into four categories: Osh1/2, Osh3, Osh4/5, and Osh6/7. With the exception of ORP2 in human and Osh4 – 7 in yeast, the PHD is found is all ORPs. OSBP and a few other ORPs also feature a unique motif comprised of two phenylalanines in an acidic tract (FFAT) that binds to vesicle-associated membrane protein (VAMP)-associated protein A (VAP-A) located at the ER (Wyles and Ridgway, 2004; Kaiser *et al.*, 2005). At their N-terminal end, a very few ORPs are known to possess ankyrin (ANK) repeats, which carry minimal information that determines their intracellular localization to late endosomes (Sedgwick and Smerdon, 1999; Johansson *et al.*, 2003). Yeast Osh3 is the only ORP that contains an N-terminal Golgi dynamics (GOLD) domain which facilitates protein-protein interactions.
Figure 3: Structural characteristics of OSBP-related protein (ORP) family. (A) Organization of domains in various human and yeast ORPs is presented here. The human ORPs are classified into six categories (indicated by Roman numerals) based on amino acid sequence homology and gene structure (Lehto et al., 2001). (B and C) Crystal structures of OSBP-related domain (ORD) from Osh4 in complex with 25-hydroxycholesterol (black, B) and phosphatidylinositol-4-phosphate (blue, C). The hydrophobic ligand-binding pocket formed by β-barrel is highlighted in green and N-terminal lid structure is coloured red (de Saint-Jean et al., 2011). (D) Solution structure of pleckstrin homology domain (PHD) of ORP 11. The PIP-accommodating site is formed by seven β-strands (cyan). (E) The solution structure of two phenylalanines in an acidic tract (FFAT) motif (orange) of
human OSBP in complex with human vesicle-associated membrane protein (VAMP)-
associated protein A (VAP-A) major sperm protein (MSP) domain (violet). Phe-359
and Phe-360 sidechains are shown as stick models in black (Furuita et al., 2010).
Protein Data Bank (PDB) IDs of the displayed structures are: B) 1ZHX, C) 3SPW,
D) 2D9X, and E) 2RR3.

(Anantharaman and Aravind, 2002). Both ORP5 and ORP8 contain C-
terminal transmembrane regions that help in anchoring the protein to the
ER membrane (Galmes et al., 2016). Within each sub-family, ORPs show a
greater than 70 % sequence identity, which hints towards their conserved
biological functionality in human and yeasts (Tong et al., 2016).

1.3.2. *The OSBP-related domain (ORD)*

The defining feature of all members of the ORP family is a conserved
C-terminal domain ORD which binds both sterols and phospholipids (Im et
al., 2005; de Saint-Jean et al., 2011). Crystal structure elucidation of Osh4, by
Im et al. in 2005, was an important milestone in OSBP research (Im et al.,
2005). In their work, they demonstrated that Osh4 (a.k.a. Kes1) consisted of
the ORD only and lacked other domains commonly found in human ORPs.
This gave evidence in favor of the largely cytosolic nature of Osh proteins
which enabled them to diffuse freely throughout the cytoplasm, unlike
Osh1, -2 and -3, which are more-or-less localized to certain compartments
owing to the presence of additional domains at their N-termini.

Osh4 contains 434 amino acids and the structure of its ORD revealed
an incomplete β-barrel, formed by seventeen β-sheets, that constitute a
hydrophobic tunnel for ligand-binding. The N-terminal subdomain (residues
30-117), containing two β-strands and three α-helices, forged the remaining
portion of the tunnel. The entrance to this hydrophobic pocket was covered by
an amphipathic α-helix attached to a flexible loop that formed a “lid”-like structure at the far N-terminus of the protein (residues 1-29).

Figure 4: Interactions of 25HC and PI(4)P with the ORD of Osh4. The molecular interactions of the 3-hydroxyl (A) and 25-hydroxyl (B) groups of 25HC and the 1-phosphate (C) and 4-phosphate (D) groups of PI(4)P with the conserved residues of Osh4-ORD are shown. The β-barrel forming the binding pocket is coloured orange and the N-terminal lid is represented in magenta. The yellow spheres (in A and B) represent water of crystallization. The colour code used for the elements in the chemical structures: C = green, O = red, P = black, and N = blue. The images are prepared in Visual Molecular Dynamics (ver. 1.9.3) from the PDB structures 1ZHX (Im et al., 2005) and 3SPW (de Saint-Jean et al., 2011).
Although the above-mentioned features are limited to yeast ORDs, a high level of sequence similarity and like biochemical properties suggest that this structural fold is well conserved over all eukaryotic species. ORDs are known to accommodate single molecules of several types of sterols and phospholipids. The most commonly investigated ligands are cholesterol, ergosterol, oxysterols, phosphatidylinositol-4-phosphate (PI(4)P), and phosphatidylserine (PS). Sterols have been reported to interact with the internal cavity of the ORD (Figure 3B) in an orientation that allows the 3-hydroxyl group to be buried at the very bottom of the β-barrel. The functional group is stabilized by water-mediated interactions with Gln-96 (Figure 4A), which forms a part of a cluster of polar residues at the underside of the tunnel (Im et al., 2005).

The remarkable absence of any direct hydrogen bonds between the alkyl side chain-substituted hydroxyl groups of oxysterols and the conserved amino acids of Osh4 makes it difficult to fully comprehend the higher affinity of ORPs for oxysterols when compared to that for cholesterol (Dawson et al., 1989a; Ridgway et al., 1992; Wang et al., 2002, 2008). While the 20- and 25-hydroxyl groups of respective oxysterols communicate with the residues at the inner surface of the lid indirectly via relatively ordered water molecules (Figure 4B), the 7-hydroxyl group of 7HC does not form any hydrogen bonds with either water or the protein (Im et al., 2005). The interaction of the alkyl side chain of sterols with the “lid” residues lead to a “closed” conformation of the protein that creates a completely sealed environment for the bound sterol during inter-membrane transport (Im et al., 2005). The recognition of the 3-hydroxyl group of sterols by the polar residues at the bottom of the ORD is not conserved among the ORP variants, which is the underlying reason for their diverse ligand specificities (Tong et al., 2016). For example, while sterol-binding is a common theme for Osh4 and Osh5, the smaller hydrophobic pocket of Osh3 doesn’t allow for the same (Tong et al., 2013).
In 2011, Antonny and co-workers discovered that PI(4)P competitively bound to the same internal binding site of Osh4 as do sterols. With the determination of the crystal structure of Osh4 in complex with PI(4)P, it was revealed that the two acyl chains of the ligand made weak non-specific interactions with the hydrophobic sterol-binding pocket, while the headgroup was recognized and stabilized by both direct and water-mediated contacts with a shallow cavity near the tunnel entrance (Figures 3C, 4C and 4D). The 4-phosphate group engages in directing hydrogen bonding with His-143 and His-144 of the OSBP signature sequence and Arg-344. The 1-phosphate group, that acts as a connecting link between the glycerol moiety and the inositol ring of PI(4)P, establishes hydrogen bonds with Lys-109 and Lys-336. The hydroxyl groups of the inositol ring were found to mediate water-based interactions with various residues of the “lid” and an α-β-loop near the tunnel entrance (de Saint-Jean et al., 2011). The study also revealed that due to steric hindrance between His-143 and the 5-phosphate group, phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) could not be accommodated by Osh4. In human OSBP, His residues at positions 524 and 525 correspond to His-143 and His-144 of yeast Osh4, respectively. Therefore, His → Ala mutation at those sites, in the ORD of OSBP-HH/AA, renders the protein non-functional in terms of its ability to extract PI(4)P from membranes (Goto et al., 2016).

Complete absence of in vitro sterol extraction and transfer activity has been observed in some ORPs. Instead, they participate in PS homeostasis by transporting the ligand from the ER to the PM in exchange for PI(4)P in vivo (Maeda et al., 2013; von Filseck et al., 2015b). One such example is Osh6. The crystal structure of its ORD in complex with PS shows much similarity with its PI(4)P-bound form. The binding pocket of Osh6 is slightly deeper than that of Osh4 and is mainly made up of hydrophobic residues. PS is known to insert one of its long acyl chains inside the tunnel while the headgroup and
the other acyl chain are accommodated around the entrance. The hallmark of PS recognition is the formation of hydrogen bonds between the carboxylate group of the PS headgroup and the α-β-loop of the “lid”, as well as with Ser-183 in the wall of the β-barrel. Other residues at the entrance engage in hydrophobic interactions with the acyl chain (von Filseck et al., 2015b). Like Osh6, mammalian ORP homologs, ORP5 and ORP10, show poorer binding to cholesterol but increased preference for PS as a second lipid for exchanging PI(4)P at membrane surfaces (Maeda et al., 2013).

1.3.3. The pleckstrin homology domain (PHD)

PHDs are found in a variety of proteins families, including, but not limited to, Ser/Thr protein kinases, GTPases, regulators of small G-proteins, cytoskeletal proteins, PI-specific phospholipase C (PI-PLC) in mammals, ORPs, ceramide kinase, etc. (Rebecchi and Scarlata, 1998). This domain is well-known to facilitate protein localization to the Golgi apparatus by targeting both phosphoinositides and the Arf family of small G-proteins, which are abundant in the membrane of that organelle (Lemmon, 2007). Many ORPs harbour this ~120 amino acid-long domain at their N-terminal end.

Characterized by a C-terminal amphipathic helix, and a PIP-binding cavity formed by two anti-parallel β-sheets (Figure 3D), this domain exhibits a low sequence conservation among various proteins due to variation in the length of the three interstrand loops connecting the seven-stranded β-sandwich (Saraste and Hyviinen, 1995). These variable loops lie at the surface of the lipid bilayer when PHD interacts with membrane-bound phosphoinositides. This was demonstrated by Lemmon and Ferguson using a hypothetical model of interaction of the PHD with a membrane-bound phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) (Figure 5), recreated
from the X-ray crystal structure of the PH domain of Dual Adapter for Phosphotyrosine and 3-Phosphoinositides (DAPP1) bound to inositol-1,3,4,5-tetrakisphosphate (Ferguson et al., 2000). The negatively charged headgroups of PIPs are stabilized by the positively charged face of the domain projected towards the membrane (Lemmon, 2007). PHDs from different proteins have varying affinities for each species of PIP, ranging from micromolar to nanomolar range (Lemmon and Ferguson, 2001). For example, the PHD of pleckstrin shows weaker binding ($K_d \approx 30 \mu M$) to PI(4,5)P$_2$ as compared to the PHD of phospholipase C-δ$_1$ (PLC-δ$_1$) ($K_d = 1.7 \mu M$) (Yagisawa et al., 1994; Lemmon et al., 1995).

Figure 5: A hypothetical model of interaction of DAPP1-PHD with membrane-bound PI(3,4,5)P$_3$. A ribbon representation of the X-ray crystal structure of DAPP1-PHD (PDB ID: 1FAO), bound to the inositol-3,4,5-triphosphate headgroup of PI(3,4,5)P$_3$ embedded in a model for a dimyristoylphosphatidylcholine bilayer, is shown. The image has been reprinted with permission from (Lemmon and Ferguson, 2001).
In human OSBP, Arg-109 and Arg-110 are known to be crucial for the recognition of PIP-bearing membranes (Levine and Munro, 1998, 2002; Ngo and Ridgway, 2009; Goto et al., 2016). Loss of basicity at these sites due to Arg → Glu mutation hampers the protein’s ability to associate with membrane via PI(4)P-PHD interaction. The OSBP-RR/EE mutant, however, retains its ability to extract PI(4)P from membranes, due to an intact and functional ORD. (Goto et al., 2016).

By interacting with various PIP species distributed throughout the endomembrane system, PHDs help in docking many LTPs to their target membranes, for example, ORPs, four-phosphate-adaptor protein 2 (FAPP2) and ceramide transfer protein (CERT) (Hanada et al., 2003; Im et al., 2005; Yamaji et al., 2008). While this domain targets Osh1, OSBP and ORP9L to Golgi, it guides ORP1L to late endosomes and ORP3, -6 and -7 to the PM (Tong et al., 2016). The overall membrane targeting of ORPs originate from a synergistic combination of PHD and other motifs such as ANK and FFAT. Furthermore, the simultaneous detection of both PI(4)P and Arf1-GTP at the Golgi by the PHD facilitate precise localization of OSBP to ER-Golgi MCSs and help in initiating PI(4)P-sterol exchange between the two organelles (Mesmin et al., 2013).

1.3.4. The FFAT motif

Many ORPs feature a consensus amino acid sequence of Glu-Phe-Phe-Asp-Ala-X-Glu that enable these cytosolic proteins to localize at the ER. This conserved motif binds to the ER resident VAP-A, which is anchored to the membrane by its transmembrane domain. This protein has three main domains: an amino-terminal major sperm protein (MSP) homology domain, a central coiled-coil domain and a carboxy-terminal transmembrane helix (Lev et al., 2008). The FFAT motif of ORPs interact with the MSP domain that
folds to form an immunoglobulin like β-sandwich structure as shown in Figure 3E. The interaction happens by electrostatic interactions of the acidic residues, flanking the phenylalanines of FFAT, with the basic residues on the surface of the MSP domain (Kaiser et al., 2005). The phenylalanines also engage in favorable interactions with the conserved hydrophobic patch on VAP-A surface (Figure 6). Furuita et al., in 2010, identified an acidic patch, made of Asp-352, Glu-353, Asp-354, Asp-355, and Glu-356, upstream of the FFAT motif. In their study, they found that this patch enhances the binding affinity of FFAT by facilitating the formation of an intermediate complex with VAP-AMSP through ionic interactions (Furuita et al., 2010).

Figure 6: Interaction of FFAP motif with VAP-A. The solution structure of two FFAT motif (green ribbon model) of human OSBP in complex with the MSP domain of human VAP-A (surface model). The Phe-359 and Phe-360 (yellow), and the flanking acidic residues (red) of OSBP_{FFAT}, are displayed as stick models. The residues on the surface of VAP-AMSP are differentiated as: acidic (red), basic (blue), polar (white) and hydrophobic (yellow). The image is prepared in Visual Molecular Dynamics (ver. 1.9.3) from the PDB structure 2RR3, solved by NMR studies (Furuita et al., 2010).
1.3.5. Biological functions of various OPRs

A number of studies have confirmed that the functions of ORPs are not limited to cellular sterol metabolism. OSBP, the founding member of this family, is known to recruit CERT to the Golgi, in the presence of 25HC. Resulting increase in the flux of ceramide to trans-Golgi facilitates sphingomyelin synthesis and release of diacylglycerol (DAG) (Perry and Ridgway, 2006). The PI(4)P-dependent recruitment of OSBP and CRET to Golgi by the PI/PC transfer protein Nir2 is responsible for regulating Golgi DAG levels and PI4P synthesis by PI4K-kinases (Peretti et al., 2008). DAG and PI(4)P are both involved in controlling vesicle biogenesis from Golgi.

Among the other members of the mammalian ORP family, the sterol sensor ORP1L acts as a scaffold in regulating the motility of late endosomes (LE) in cells (Rocha et al., 2009). Under low cholesterol condition, ORP1L facilitates the formation of ER-LE MCSs by interacting with the ER-resident VAP. This, in turn, causes movement of LEs to the plus end of microtubules due to removal of associated dynein/dynactin motor proteins from LEs. The process is prevented under high cholesterol conditions.

ORP9, a member of the subfamily V, modulates phosphorylation of Akt/protein kinase B at its phosphoinositide-dependent kinase-2 (PDK-2) phosphorylation site under the regulatory control of Mechanistic Target of Rapamycin (mTOR) kinase (Lessmann et al., 2007). This, in turns, affects glucose metabolism and other processes that have significant impact on cell survival and cell cycle progression. A recent study by Sahir and co-workers have demonstrated the anti-proliferative action of a class of natural products, called ORPphillins, in tumor cells lacking p21 (Burgett et al., 2011). These compounds perturb the sub-cellular distribution and induce proteasomal degradation of ORP4L by occupying the same binding pocket of the ORD that accommodates 25HC. The implications of the critical role of ORP4L in Ca2+
regulation, cell proliferation and survival, make it a potential target for anti-cancer therapeutics.

ORP8, a close homolog of ORP5, is known to anchor at the ER membrane and nuclear envelope by virtue of its carboxy-terminal transmembrane domain. By interacting with nucleoporin 62 (Nup62) (Béaslas et al., 2012), a component of the nuclear pore complex, ORP8 regulates the nuclear transport which, in turn, impacts the functioning of SREBPs and LXRs. Likewise, physical interactions of ORP3 and ORP7 with the small GTPase R-Ras (Goldfinger et al., 2007; Lehto et al., 2008), are known to regulate signaling pathways associated with cell adhesion and migration, especially during malignant growth. Another member of the ORP family, ORP2 acts as a sterol receptor on cytoplasmic lipid droplets (LD) in adipocytes, and the ORP2-LD association is inhibited by 22HC, thereby indicating a role of this protein in neutral lipid metabolism (Hynynen et al., 2009).

1.3.6. Proposed mechanisms of monomeric exchange and bidirectional transport of lipids by ORPs

Elucidation of the crystal structure of the yeast ORP named Osh4 (Im et al., 2005) and some recent mechanistic studies (de Saint-Jean et al., 2011; von Filseck et al., 2015a) have firmly established that Osh4 acts as a PIP-sterol exchanger and transports ergosterol from the ER to trans-Golgi while bringing phosphoinositide, such as PI(4)P, back to the ER (Figure 7). Despite the absence of a PHD in Osh4, this bidirectional shuttling is achieved by the unique ability of the conserved sterol-binding/transfer domain (ORD) to effectively bind sterol as well as PI(4)P with similar affinity. This explains why Osh4 was able to rescue yeast strains with deleted Osh gene family (Beh and Rine, 2004). By exchanging sterol for PI(4)P, Osh4 was able to execute a
net transfer of sterol to the PM and Golgi, while the mutants with impaired ability to sequester PI(4)P, such as K336A and H143A/H144A, failed to do so (Im et al., 2005).

The yeast ORPs that lack the VAP-A binding FFAT motif, such as Osh4, possess two membrane binding surfaces in their ORDs. The one near the mouth of the sterol-binding pocket is responsible for PI(4)P/sterol exchange. The site distal to the binding pocket binds certain PIPs, such as PI(4,5)P$_2$ of a closely apposed acceptor membrane and regulates sterol extraction and transport from the donor membrane (Schulz et al., 2009). Osh4 is known to use the PI(4)P gradient between the Golgi and the ER, created by the ER-resident phosphoinositide phosphatase Sac1 and phosphatidylinositol 4-kinase Pik1 at the trans-Golgi, as an energy source for the transport of oxysterols against their concentration gradient (von Filseck et al., 2015a). Bankaitis and associates showed that the expression of Osh4 was lethal for temperature-sensitive yeast strains lacking the essential gene encoding Sec14, a PI/PC transfer protein (Li et al., 2002). However, the deletion of Osh4 enabled the Sec14-deficient yeast strains to bypass their phenotype and survive. Discovery of the PI(4)P-sterol exchange mechanism by Antonny and co-workers now provides a fitting explanation for this observation (de Saint-Jean et al., 2011). The cell survival in OSH4-SEC14 double deletion mutants is believed to be caused by the reduced backward transport of PI(4)P from the trans-Golgi to the ER. This results in the much-needed preservation of the limiting amount of PI(4)P synthesized by Pik1 when the anterograde transport of PI by Sec14 is compromised.

Antonny's group has also done extensive research on mammalian OSBP-mediated transfer of sterols and PIPs between donor and acceptor liposomes using Förster resonance energy transfer (FRET) (Mesmin et al., 2013). Their work has established that mammalian OSBP can simultaneously interact with ER membrane protein VAP-A via its FFAT
motif and Golgi-specific PI(4)P and ADP-ribosylation factor 1 (Arf1)-bound GTP, via its PHD, causing membrane tethering at the MCS. This enables sterol transfer from ER to Golgi followed by back transfer of PI(4)P by the ORD (Figure 7). Finally, PI(4)P that is removed from the Golgi is hydrolyzed in the ER by the integral membrane phosphatase Sac1 (Kim et al., 2013). The energy released by the hydrolysis drives sterol transfer and allows negative feedback when PI(4)P becomes limiting.

Figure 7: Proposed models of PIP-sterol exchange by ORPs. Single ORD in yeast Osh4, binds sterols and PI4P within the same pocket and facilitates exchange of these two ligands at membrane surfaces. This results in an overall flux of sterols from the ER to Golgi, and movement of PIP in the opposite direction. In humans and other mammals, OSBP localized at a MCS first binds ER-resident VAP-A by FFAT motif, followed by extraction of oxysterol from the ER (fully shaded ORD) and concomitant activation of PHD. Activated PHD binds PIP at the non-ER membranes and facilitates efflux of oxysterol from ER to target organelles (partially shaded ORD), such as the Golgi and PM while bringing back PIP to the ER.
It became apparent over a decade ago that localization of OSBP to various cellular organelles, such as the Golgi, ER and PM, is largely determined by two factors: the phosphorylation state of OSBP and the sterol content of cells (Storey et al., 1998). According to Ridgway and co-workers, depletion of cholesterol causes dephosphorylation of OSBP, which in turn localizes the protein at Golgi that harbours the phosphorylation machinery. In one of their recent studies, OSBP mutants mimicking the partial or complete phosphorylation state of the protein were designed to investigate the effects of phosphorylation on the sterol binding and transferability of OSBP (Goto et al., 2012). Two serine-rich motifs were identified to regulate OSBP activity at the ER. One was formed by Ser-381, -384, -387, -388 and -391 (site 1 - located between the ORD and FFAT), and another was composed of Ser-192,195,200 (site 2 - located immediately upstream of the C-terminal end of the PHD). A site 1 phosphomimetic mutant, with Ser → Glu mutations at all five serine residues, demonstrated higher sterol binding and extraction, but diminished transfer activity compared to the wild-type protein. These mutations, however, did not affect the PI(4)P binding/transfer capability of OSBP.

Despite the wealth of knowledge gathered about the mechanism of lipid transport by the members of the ORP protein family and their biological role, the precise nature of the association of ORPs with organelar membranes and the factors modulating such interactions requires further investigation. Given the abundance of in vitro fluorescent and radiolabel-based assays with somewhat intricate and ambiguous design, a label-free analytical approach towards direct examination of protein-lipid bilayer interactions, using simple membrane systems, holds promising potential.
1.4. Research Objectives and Outline

The primary goal of this project was to characterize the ability of OSBP to recognize membrane lipids by studying its association with surface-immobilized PC bilayers using dual polarization interferometry (DPI) and monitoring ligand transfer with the help of a fluorescent sterol analog. My objectives included:

Phase I

i. Expressing, isolating and purifying recombinant OSBP using baculovirus expression vector system (BEVS) and conducting a series of membrane-binding assays on the DPI instrument by incorporating varying mol% of sterol ligands in the adsorbed bilayers. To investigate if (and how) wtOSBP interacted with the membranes based on the ligand concentration, I chose to study two sterols, namely, cholesterol and its most commonly used oxygenated derivative, 25-hydroxycholesterol.

ii. Performing the same assays, with adsorbed bilayers containing varying molar quantities of PI(4)P and validating the importance of PHD in PIP-recognition by the use of PHD mutant of OSBP (OSBP-RR/EE).

iii. Investigating the ability of OSBP to extract PI(4)P from immobilized membranes by comparing the behaviour of the wild-type protein with its ORD mutant (OSBP-HH/AA) that is known to possess unaltered PIP-recognition but impaired PIP-extraction capability.
Phase II

iv. Studying the kinetics of monomeric sterol binding by wtOSBP and OSBP-HH/AA using 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesten-3β-ol – a fluorescent sterol analog abbreviated as 22-NBD-cholesterol.

v. Examining the transport of sterol by wtOSBP to vesicles containing PI(4)P by monitoring Förster resonance energy transfer (FRET) between 22-NBD-cholesterol and N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (abbreviated as N-Rhod-PE).

The subsequent sections of this chapter provide a general overview of the technological approaches utilized in this research to meet the objectives listed above.

1.5. Baculovirus Expression Vector System (BEVS)

In vitro characterization and structure determination of proteins require their large-scale production in lab. In vivo expression systems follow traditional approaches wherein either prokaryotic or eukaryotic cells are transfected with DNA vectors engineered to carry the gene of interest and cultured in appropriate media to transcribe the gene and translate it into protein. This is typically followed by cell lysis, extraction and subsequent purification of the desired protein (Gräslund et al., 2008). On the other hand, in vitro (or cell-free) techniques utilize extracts of whole cells that are capable of transcription, translation and to some extent, post-translational
modifications of expressed proteins when supplemented with cofactors, nucleotides, the specific gene template and chaperones (Rosenblum and Cooperman, 2014).

Each expression system offers selective advantages and disadvantages, particularly for the production of proteins larger than 100 kDa. Prokaryotic systems, such as *E. coli*, are known for rapid growth in culture and fast recombinant protein production, especially with the T7-based polymerase system. Expression of multi-domain eukaryotic proteins in *E. coli* renders them non-functional due to lack of cellular machinery required for proper protein folding and appropriate post-translational modifications. Also, the elevated yields make the proteins insoluble as inclusion bodies that are extremely cumbersome to recover and often require harsh treatments which pose the risk of protein denaturation. However, novel methods, involving milder solubilization techniques, have surfaced in recent years (Singh *et al.*, 2005, 2015). Mammalian host cells are the common choice for the production of larger eukaryotic proteins but this expression platform suffers from low yields, high production costs and is laborious and time-consuming. Cell-free techniques are also not sustainable for large scale production.

In the mid-80s, insect baculoviruses became widely popular as novel vectors for heterologous expression of foreign genes in cultured insect cells and larvae (Clem and Passarelli, 2013). Owing to a host range restricted to lepidopteran insects (such as moths and butterflies), complete lack of pathogenicity in vertebrates and plants, capability of proper post-translational modifications, growth in serum-free media and ease of scaling up, these viruses have become one of the most versatile and powerful eukaryotic vector systems for accurate recombinant gene expression. With the advent of specialized media, transfection reagents and chimeric vectors, BEVS technology has found several mainstream applications ranging from
structural and functional studies to development of vaccines and diagnostics (van Oers, 2011).

1.5.1. Structural assembly of baculovirus

The double-stranded, circular and supercoiled genome of a baculovirus spans approximately 80 – 180 kbp and is encapsulated in a large, rod-shaped capsid. Known to be one of the most prominent pathogens to obliterate vermian populations, these viruses have been identified particularly in insects of the order Lepidoptera (Clem and Passarelli, 2013). Baculovirus exists in two different forms of infectious particles (virions): budded virus (BV) and occluded/occlusion-derived virus (ODV).

In infected larvae, budded virions are responsible for tissue-wide transmission of infection in vivo. The second-type of virions are occluded in large proteinaceous bodies called occlusion bodies (OBs), which are responsible for vertical transmission of infection from one infected larva to another. The ODVs are assembled entirely in the nucleus and, based on the number of virions packed in OBs, baculoviruses are broadly classified as granuloviruses, which are characterized by the presence of only one virion in each granular OB, or nucleopolyhedroviruses (NPVs), which produce large polyhedral OBs that carry hundreds of virions. In certain species of NPVs, ODVs can carry virions harbouring either single or a variable number of nucleocapsid units. Therefore, these NPVs are unofficially categorized into two subtypes, namely, single nucleopolyhedroviruses (SNPVs) and multiple nucleopolyhedroviruses (MNPVs). The two baculoviruses mostly widely used in the field of biotechnology as vectors for recombinant protein production are Autographa californica (alfalfa looper) multiple nucleopolyhedroviruses (AcMNPVs) and Bombyx mori (silkworm) nucleopolyhedroviruses (BmNPVs) (van Oers, 2011; Clem and Passarelli, 2013).
1.5.2. *Baculovirus* life cycle

*Baculoviruses* undergo both lytic and occlusive journeys during their life cycles. However, these events occur independently of each other over four distinct phases: immediate-early, delayed-early, late and very late. In the very beginning of the cycle, ODVs are ingested by insect larvae from plant surfaces. Upon entering the mid-gut of the feeding worm, the crystalline proteinaceous matrices of OBs dissolve in the alkaline milieu, thereby releasing BVs that attach, penetrate and fuse with the epithelial cells of microvilli. This marks the commencement of the early phase which is a preparatory phase for viral DNA replication. Following uncoating of viral particles, host gene expression is shut off and the viral immediate-early genes are transcribed by host-encoded RNA polymerase II (Rohrmann, 2013c). This, in turn, initiates the transcription of delayed-early genes which encode proteins essential for DNA replication, such as DNA polymerase, helicase viral RNA polymerase, etc. (Rohrmann, 2013b).

Late phase, also known as the virus structural phase, features viral DNA replication and expression of late genes that code for nucleocapsid and viral coat proteins. These proteins are responsible for virion assembly and budding. It is during this phase that new BVs are produced (Rohrmann, 2013a). Their release by exocytosis and concomitant uptake by adjacent cells via endocytosis result in the horizontal spread of the infection.

The very late phase is the viral occlusion protein phase wherein two major proteins, polyhedrin and P10, are produced in high quantity. Polyhedrin assembles to form the crystalline matrices of OBs within which virus particles synthesized in the nucleus are embedded to give rise to ODVs (Rohrmann, 2013d). The release of ODVs from the nucleus is facilitated by P10 and is followed by cell lysis that marks the end of the infection cycle.
Upon the subsequent death of the insect larvae, ODV polyhedra are released onto leaves and plant surfaces for vertical transmission of infection.

1.5.3. Development of baculovirus expression vectors

Direct cloning of a foreign gene, by means of a bacterial plasmid, into the 134 kbp AcMNPV genome is not a viable option. In 1983, Smith and Summers patented a method that involved homologous recombination between the baculovirus genome and the foreign gene contained in a transfer plasmid, under control of the P10 or polh promoter (Smith et al., 1983). The isolation of recombinant baculoviruses from the parental wild-types required sequential rounds of plaque purification, which was an extremely cumbersome process. In order to improve the success of recombination, Kitts et al. introduced linearized viral DNA in 1990 (Kitts et al., 1990). This technique resulted in ~30 % increase in the recovery of recombinants.

The following decade witnessed several improvements, but a major breakthrough was achieved with the development of bacterial artificial chromosomes containing the AcMNPV genome (Luckow et al., 1993). Today, these vectors, termed bacmids, are introduced in E. coli (DH10β) and the eukaryotic gene of interest is incorporated into the bacmid by site-directed transposition using Tn7-recombinase (Figure 8). After PCR verification of the transgene, the recombinant bacmid is isolated to transfet insect cell lines. BV particles are propagated from the cultures and used to infect new cells. Several rounds of infection produce high-titre seed stocks of baculovirus for recombinant protein production. The baculovirus stocks, used in my work for the expression of OSBP and its mutants, were received as a gift from Dr. Neale Ridgway and his co-workers from Dalhousie University, Nova Scotia, Canada.
1.5.4. Choice of host cell lines

Among several established insect cell lines, Sf9 and Sf21 are the two most widely used cell lines that are highly susceptible to AcMNPV infection (Li et al., 1996; Kost et al., 2005; Schneider and Seifert, 2010). Originating from the ovarian tissues of Spodoptera frugiperda (fall armyworm) larvae, both these cell lines are highly adaptable to adherent as well as suspension cultures and can be distinctly identified by their enlarged and uniformly round morphology, enlarged nuclei, detachment from surface and inability to proliferate when infected by baculoviruses. Sf9 and Sf21 can be adapted to grow effectively in serum-free media at 27 ± 1 °C and generally do not require
antibiotic and CO₂ supplementation. These favorable attributes make them the ideal host cells for use in BEVS technology.

1.6. Dual Polarization Interferometry (DPI)

Ever since the commercialization of Surface Plasmon Resonance (SPR) by Biacore® (GE Healthcare) in 1990 (Karlsson et al., 1991), the discipline of surface analytical chemistry has revolutionized research in the areas of biophysical, biomedical and pharmaceutical sciences. With advances in the developmental design of various biosensors, a plethora of investigations ranging from rapid screening of putative drug candidates to identification of harmful food toxins, have become a reality (Malhotra et al., 2014; Prével et al., 2014; Sahdeo et al., 2014). Today, surface techniques offer label-free (fluorescent, chemical or radiolabeled tags), user-friendly and real-time data collection. This enables the study of binding of small molecules to surface adsorbed biomolecules, protein-protein, protein-membrane and receptor-ligand interactions, nucleic acid hybridization, kinetics of enzyme-substrate association and dissociation, protein crystal growth and more (Daghestani and Day, 2010; Boudjemline et al., 2011). One such technology, named Dual Polarization Interferometry (DPI), was commercially made available in 2000 by Farfield Group Ltd (Swann et al., 2004). This approach provides a unique avenue for studying the solution-phase binding of a macromolecule (or small compound) with a physically immobilized biomolecular layer over the surface of a plain or chemically-derivatized chip. In addition, DPI provides an excellent platform for configuring parameters that define bilayer quality (such as appropriate thickness, refractive index, mass deposited per unit area, etc.) which aids in maintaining identical conditions for all protein adsorption studies and improves reproducibility and reliability of data acquisitions.
1.6.1. The working principle of DPI

The operation of DPI is based on a pioneering experiment performed in 1801 by an English physicist named Thomas Young, who demonstrated the interference of light waves. By allowing a beam of sunlight to pass through two narrowly placed slits in a sheet of paper, he noticed that a characteristic “dark and light pattern” was produced by the rays diffracting from the slits onto a screen held several meters away. In theory, when light waves from two coherent monochromatic sources interfere, they produce a pattern of alternate dark and light spots which is not visible in space since there is nothing substantially large in the air to reflect the light to our eyes. However, when projected onto a screen, this distinct pattern becomes visible. The locations where the light waves constructively reinforce each other appear abnormally bright and are referred as antinodes and the locations where the light waves destructively eliminate each other appear abnormally dark and are hence referred as nodes. Through his “double slit” experiment, Young derived an equation for calculating the wavelength of the light originating from the source and resurrected the century-old theory that light has a wavelike nature.

1.6.2. Overview of DPI system components and data acquisition

The DPI instrument derives its light source from a helium-neon laser that emits at a constant wavelength of 632.8 nm. The emitted light passes through a polarizer and is fed into a biosensor that consists of two waveguides stacked on top of each other with a layer of cladding sandwiched between them (Figure 9). The setup mimics Young’s “double slit”. The waveguides are made of silicon oxynitride and the light waves entering them remain coherent (with constant phase difference and frequency) and confined within well-defined boundaries.
Figure 9: Operating principle of dual polarization interferometry. A schematic diagram of DPI instrument is shown. A laser-generated monochromatic light passes through a polarizer (not shown for clarity) and splits into two coherent beams upon entering the biosensor. The light waves diffracting from the biosensor waveguides produce a characteristic interference pattern. Adsorption of biomolecules on the sensing surface and subsequent changes in the refractive index cause phase shifts in the double polarizations (TM and TE) of the interfering light waves. These signals are then resolved by the instrument into physical variables depending on the applied algorithm (Swann et al., 2004).

Upon adsorption of a biomolecular layer (for example, a phospholipid bilayer), the velocity of light traveling through the top waveguide changes and acts as a “sensor” while the one traveling through the bottom waveguide
remains unaltered and hence, serves as a “reference”. The emergent light waves, no longer in phase, diffract and interfere with each other at “far field”, thereby producing a series of dark and light fringes. The spatial separation of nodes and antinodes is defined by the phase difference between the two interfering light waves emerging from the ends of the waveguides.

The position of the interference fringes shifts when the light traveling through the top waveguide changes further due to addition and interaction of proteins to the immobilized bilayer. These movements are captured by a camera and the changes in the transverse magnetic (TM) phase are deduced from the distances moved by the fringes. A second polarization of the light, namely transverse electric (TE), is directed at an angle perpendicular to the TM and thus provides an independent second measurement of protein association/dissociation. The DPI technology is capable of translating the raw data obtained from these two measurements into comprehensible physical variables such as thickness, specific mass, and density of the protein layer (Swann et al., 2004).

### 1.7. Förster resonance energy transfer (FRET)

A number of cholesterol analogs are used to study and visualize the distribution of sterols in intracellular membranes. While most of these analogs are fluorophore-labeled (such as BODIPY, NBD, dansyl, etc.), there are some variants of cholesterol which carry conjugated double bonds within the steroid ring system of cholesterol. This makes them fluoresce in the near UV-region of the spectrum (Maxfield and Wüstner, 2012). Some examples are dehydroergosterol (DHE) and cholestatrienol (CTL). The chemical structures of these intrinsically fluorescent sterols closely resemble that of cholesterol.
and therefore do not perturb ligand binding to the ORD, unlike the bulky fluorescent groups such as BODIPY and NBD (Figure 10).

![Dehydroergosterol (DHE)](image1)
![Cholestatrienol (CTL)](image2)
![6-Dansyl-cholesterol](image3)
![22-NDB-cholesterol](image4)
![23-BODIPY-cholesterol](image5)

**Figure 10: Structures of commercially available fluorescent cholesterol analogs.** Fluorescent cholesterol reporters commonly used for cholesterol binding/transfer assays: DHE, CTL (cholesta-5,7,9(11)-trien-3β-ol), 6-dansyl-cholesterol, 22-NBD-cholesterol (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnorcholesterol-5-en-3β-ol), and 23-BODIPY-cholesterol (23-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazas-indacen-8-yl)-24-norchol-5-en-3β-ol).

DHE and CTL, however, suffer from the disadvantage of low fluorescence brightness and poor environmental sensitivity (Chong and Thompson, 1986; Schroeder et al., 1988; Hyslop et al., 1990). NBD groups are
known to display a high degree of environment sensitivity (Haldar and Chattopadhyay, 2013). They are usually characterized by a markedly longer fluorescence lifetime and higher intensity of fluorescent emission in hydrophobic media such as membranes as compared to a polar surrounding. NBD-labeled cholesterol analogs, especially the alkyl side-chain substituted ones, tend to localize inside the membrane’s interior or the hydrophobic pocket of LTPs, such as ORPs.

Figure 11: Chemical structures and spectral overlap of 22-NBD-cholesterol and N-Rhod-PE. The chemical structures of 22-NBD-cholesterol and N-Rhod-PE are shown. The region in red represents the NBD fluorophore and the one in blue represents the Lissamine™ Rhodamine B fluorophore. Scans of 22-NBD-cholesterol and N-Rhod-PE fluorescence from 300 – 700 nm are shown here. The overlapping region of the emission spectrum of NBD and the excitation spectrum of Rhodamine is represented by the shaded region. The arrows indicate the excitation wavelength (red) and emission wavelength (blue) used for FRET-based ligand transfer assay.
Förster resonance energy transfer (FRET) is a physical phenomenon wherein a donor fluorophore absorbs energy of incident photons and transfers the energy of its excited state to a nearby acceptor fluorophore. As a result, the donor fluorescence gets quenched along with a decrease in the lifetime of its excitation and a concomitant increase in the intensity of acceptor fluorescence, which always emits at a longer wavelength than that of the donor (Hussain, 2012). The fluorescence intensity decreases with an increase in the average spatial separation of the probes (Johnson and Spence, 2010). The excitation and emission spectra of a FRET donor-acceptor pair is shown in Figure 11.

Several molecular probes are commercially available today for FRET-based analysis of ligand transfer by proteins (de Saint-Jean et al., 2011; Mesmin et al., 2013) and monitoring protein-protein or protein-lipid interactions (Margineanu et al., 2016). The choice of the FRET pair, therefore, completely hinges on the nature of the system to be investigated and the behaviour of the probe molecule in the environment in which the desired assay is to be performed. For this project, I chose to use the NBD-Rhodamine pair. FRET occurs when OSBP-bound 22-NBD-cholesterol is delivered into the membrane bilayer of an acceptor vesicle containing N-Rhodamine-phosphatidylethanolamine. The rate of transfer is typically monitored by recording over time the intensity of rhodamine emission at ~581 nm, a consequence of energy transfer when NBD is excited at ~469 nm.
Chapter 2

Methodology

This section gives an overview of the experimental journey of my research beginning with the heterologous expression of OSBP and its mutants in insect cell lines, followed by their isolation, purification, biochemical characterization, ultimately culminating in the study of OSBP’s ability to recognize, bind and transfer specific sterol and phosphoinositide species in solution as well as artificial phospholipid membranes. The protocols and materials utilized for the development of analytical assays have been documented in this chapter, along with the selection of mathematical models to explain the ligand-modulated lipid association and dissociation of OSBP, applicable for both label-free and fluorophore-based studies.
2.1. Culture of host cells (Sf21)

Sf21 (Life Technologies, Burlington, ON), a suitable host cell line developed from the ovaries of fall armyworm (Spodoptera frugiperda), was grown as a stable monolayer in Gibco® Sf-900 III serum-free medium (Life Technologies, Burlington, ON) supplemented with 5 % FBS (Sigma-Aldrich Canada, Oakville, ON) at 27 ± 1 °C in a non-humidified, ambient air-regulated incubator. The following antibiotics were added to prevent contamination by gram-positive and gram-negative bacteria as well as fungal infections: 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B deoxycholate (Sigma-Aldrich Canada, Oakville, ON). When the culture reached ~90 % confluency, cells were passaged at a split ratio of 1:5 in T-25 tissue culture flasks every 72 – 96 h. The seeding density was ~3 × 10^4 viable cells/cm^2. The confluency and viability were routinely monitored by observations under a light microscope and dye-exclusion assay, respectively. A 0.4% buffered solution of trypan blue (Sigma-Aldrich Canada, Oakville, ON) enabled direct identification and enumeration of live (unstained) and dead (blue) cells in Sf21 cultures.

Cell densities were measured by an improved Neubauer counting chamber and the values were routinely logged prior to the propagation of a new generation, according to the format given in Figure 12. Cultures were discarded after passaging for 20 – 25 generations and new cultures were started with freshly-thawed cells. In order to develop master stocks of cells for future use, healthy-growing Sf21 cells, showing > 90 % viability, were suspended in freezing medium, which was prepared by mixing conditioned and fresh growth media in 1:1 ratio, containing 10 % FBS and 7.5 % dimethyl sulfoxide (DMSO; Life Technologies, Burlington, ON). Cells were then aliquoted in 1.5 mL volumes, at a density of 1 × 10^7 cells/mL, in cryo-vials. The vials were cooled at a standard rate of -1 °C/min in Mr. Frosty™ freezing
container (Thermo Fisher Scientific, Mississauga, ON) before storing in liquid nitrogen vapor phase (-140 to -180 °C).

<table>
<thead>
<tr>
<th>Passage Number</th>
<th>Date</th>
<th>Cell Density (cells/mL or cells/cm²)</th>
<th>Viability (% of total # of cells)</th>
<th>Comments (split ratio, cell morphology, media lot #, etc.)</th>
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**Figure 12: Format of log for maintaining Sf21 cell line.** A typical format of log, maintained regularly during the propagation of Sf21 cell line, is shown. The date of initiation of culture from a frozen master stock, initial cell density, and net composition of the working media (including amounts of added antibiotics and serum) were also recorded.

### 2.2. Expression of OSBP using recombinant baculoviruses

After 3 – 4 passages of monolayers, the cells were transferred to shaker flasks at a seeding density of \(\sim 5 \times 10^5\) cells/mL and incubated at 27 ± 1 °C and 130 rpm in a non-humidified, ambient air-regulated incubator. They adapted to suspension after 2 generations. The culture, while in its logarithmic growth phase, was infected with AcMNPV baculovirus at a density of \(1.8 \times 10^6\) cells/mL and desired multiplicity of infection (MOI). The baculovirus constructs harbouring cDNA of C-terminal 6×His-tagged wtOSBP, OSBP-HH/AA and OSBP-RR/EE mutants were generously gifted by Dr. Neale Ridgway (Dalhousie University, Halifax, NS). The infected cells were incubated in the dark under the same growth conditions as mentioned above. The culture was monitored for signs of infection, such as increased diameter and larger nuclei, over a period of 48 – 72 h. In light of the knowledge that baculoviruses possess lytic capabilities, it was considered extremely important to optimize the MOI and monitor cell viability during the infection period. It helped to ensure that cells were harvested before they were rendered non-viable due to prolonged infection. After infecting two
batches of Sf21 cultures, one for 72 h at an MOI of 0.1 and another for 48 h at an MOI of 0.2, protein expression levels were compared (see Results, Section 3.1.3) and based on the results, MOI of 0.2 was used for subsequent expressions.

2.3. Isolation of OSBP by IMAC

Sf21 cells were pelleted at 10,000 × g and frozen overnight at -80 °C, 48 – 72 h post-infection. They were subjected to mechanical disruption at 4 °C by passing through 18 G and 23 G needles and homogenizing by sonication in lysis buffer: 20 mM Tris-HCl (pH = 7.4), 150 mM NaCl, and 1% Triton X-100. 10 mL lysis buffer was used per g of cell pellet. In order to prevent unwanted proteolysis, a suitable EDTA-free cocktail of inhibitors for serine, cysteine and aspartic proteases (Roche Diagnostics, Laval, QC) was used along with DNase I (Fisher Scientific, Ottawa, ON) for removal of nucleic acids. The extracted intracellular proteins were subjected to immobilized metal affinity chromatography (IMAC) for isolation of recombinant 6×His-tagged OSBP.

The stationary phase was made up of Profinity™ IMAC resin (Bio-Rad Laboratories Canada, Mississauga, ON) that contained Bio-Rad’s proprietary UNOsphere™ beads coupled with an iminodiacetic acid (IDA) linker. Prior to use, the resin was coupled with a divalent metal cation (in this case, Co²⁺) by incubating in 2 M CoCl₂ (pH = 4.6). The tridentate IDA ligand gives rise to an octahedral metal coordination complex with imidazole sidechains of the polyhistidine tag (Figure 13). This architecture allowed strong and highly selective binding of recombinant 6×His-OSBP and its mutants. Therefore, the use of EDTA was avoided because of its known ability to chelate with the Co²⁺ ions and disrupt binding of the polyhistidine-tagged proteins to the IMAC column. The clarified lysate was spun down at 40,000 × g and the
supernatant was incubated overnight at 4 °C with Co\(^{2+}\)-IDA resin; this was packed into a high-resolution Bio-Scale\(^{TM}\) MT column (Bio-Rad Laboratories Canada, Mississauga, ON). The chromatography process was executed in a semi-automated fashion on the BioLogic DuoFlow\(^{TM}\) FPLC system (Bio-Rad Laboratories Canada, Mississauga, ON).

**Figure 13: Capturing of His-tagged proteins.** The underlying chemistry of capturing polyhistidine-tagged protein by cobalt-iminodiacetic acid (Co\(^{2+}\)-IDA) resin during immobilized metal affinity chromatography (IMAC) is shown. The imidazole groups of His form an octahedral coordination complex with Co\(^{2+}\) along with carboxyl groups and nitrogen atom of IDA linker.
During the operation, the IMAC column was first rinsed with the running buffer: 50 mM NaH$_2$PO$_4$ and 300 mM NaCl (pH = 7.4) at 12 – 15 psi and a flow rate of 4 mL/min. Proteins containing variable number of His residues bound with the resin via weak interactions and were effectively removed by a wash with the running buffer containing 20 mM imidazole. Recombinant OSBP was eluted as 4 mL fractions under the same flow conditions with elution buffer: 50 mM NaH$_2$PO$_4$, 300 mM NaCl and 200 mM imidazole (pH = 7.4). Compared to a gravity-based chromatography setup, semi-automated low-pressure FPLC system provided several advantages, such as, reduced time of operation, increase in protein yield, ease of scaling-up, and flexibility of fine-tuning the process by adjusting buffer compositions in real-time. A summary of a batch purification of wtOSBP using IMAC-FPLC has been provided in the Results, Section 3.1.2 (Figure 17).

### 2.4. Quantification of OSBP

OSBP was quantified by the Bradford protein quantification method (Bradford, 1976). 50 µL of purified OSBP was incubated at room temperature with a 1 mL mixture of 50 % (w/v) Coomassie Brilliant Blue G-250 dye, 25 % (v/v) methanol and 50 % (v/v) phosphoric acid, commonly known as the Bradford reagent (Sigma-Aldrich Canada, Oakville, ON). The absorbance of the mixture at 595 nm was measured by a UV-Vis spectrophotometer and its concentration was determined by the help of a standard curve obtained by plotting the absorbance values of a series of BSA standards against their pre-determined concentrations. Protein concentration estimated using the Bradford method was cross-checked with the values calculated by the Beer’s Law using the relationship:

\[
A = \varepsilon cl
\]  
(eq. 1)
In this equation, $A$ stands for absorbance of the protein solution measured at 280 nm, $\varepsilon$ represents molar extinction coefficient of OSBP at 280 nm ($122270 \text{ M}^{-1} \text{ cm}^{-1}$), $c$ denotes concentration of protein in the sample, and $l$ defines the length of the path traveled by the light (1 cm, for the cuvettes used in this study).

2.5. Molecular weight analysis of OSBP by SDS-PAGE

The molecular weight of purified OSBP was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Based on the estimated protein concentrations, approximately 3 – 5 µg of crude and clarified cell lysate, unbound protein fraction from IMAC and purified protein fraction(s) were incubated for 5 min at 90 °C with the Laemmli sample buffer: 50 mM Tris-HCl, 2 % SDS, 6 % glycerol, 1 % β-mercaptoethanol, and 0.004 % bromophenol blue (pH = 6.8) and loaded onto a precast Mini-PROTEAN® TGX Stain-Free™ 10 % polyacrylamide gel (Bio-Rad Laboratories Canada, Mississauga, ON). Alternatively, hand-cast gels were also used from time to time for routine analyses. The resolving gel was prepared by mixing the following constituents in volumes determined by the size and quantity of the gels to be cast: 40% aqueous solution of acrylamide/bis-acrylamide, resolving buffer: 1.5 M Tris-HCl (pH 8.8), 10% (w/v) sodium dodecyl sulfate (SDS), freshly prepared solution of 10% (w/v) ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). For preparing the stacking gel, the resolving buffer was replaced by the stacking buffer: 0.5 M Tris-HCl (pH 6.8) in the above recipe.

The protein samples, denatured by the SDS in the Laemmli buffer and bearing uniform charge-to-mass ratio, were separated based on size by applying a voltage of 150 mV across the gel submerged in the electrophoresis
Chapter 2 | METHODOLOGY

tank filled with the SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1 % SDS (pH = 8.3) for 1 – 1.5 h. The protein bands in the stain-free gels were visualized directly by the Gel Doc™ EZ gel documentation system (Bio-Rad Laboratories Canada, Mississauga, ON) while the hand-cast gels were stained overnight with a 9:1 mixture of ProtoStain™ colloidal Coomassie Brilliant Blue G-250 (National Diagnostics, Atlanta, GA) and ethanol followed by de-staining with distilled water. The molecular weight of OSBP was determined by comparing the resolved bands with Precision Plus Protein™ standards (Bio-Rad Laboratories Canada, Mississauga, ON).

2.6. Immunodetection of OSBP by Western blot

Identity of OSBP in the eluted fractions from IMAC was further confirmed by Western blot. Unstained polyacrylamide gel from SDS-PAGE and nitrocellulose or polyvinylidene fluoride (PVDF) membrane were primed by soaking in transfer buffer: 25 mM Tris, 192 mM glycine, and 20 % methanol (pH = 8.3) for 10 min (Towbin et al., 1979). The gel was sandwiched with the membrane, between filter papers soaked in transfer buffer. The cassette was left overnight at room temperature for efficient protein transfer.

Next day, the vacant sites on the membrane were blocked to prevent nonspecific antibody binding using TBST blocking buffer: 10 % nonfat dry milk, 10 mM Tris, 150 mM NaCl, 0.05 % Tween® 20, (pH = 8) and rinsed with TBST wash buffer: 10 mM Tris, 150 mM NaCl, and 0.05 % Tween® 20 (pH = 8). The membrane was then probed with mouse anti-OSBP monoclonal IgG₁ primary antibody (dilution 1:200; Santa Cruz Biotechnology, Dallas, TX) for 1 h followed by rinsing with TBST wash buffer and incubation with anti-mouse IgG-horseradish peroxidase (HRP) conjugate secondary antibody (dilution 1:2500; Promega, Sunnyvale, CA) for 30 min. After a final rinsing with TBST
wash buffer, OSBP was visualized as distinct band(s) by exposing the membrane to liquid HRP-substrate 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich Canada, Oakville, ON) for 3 – 5 min.

2.7. Preparation and size-determination of unilamellar lipid vesicles

The following phospholipids and sterols were used for the membrane-binding assays using DPI: 1,2-dioleoyl-<i>sn</i>-glycero-3-phosphocholine (DOPC), L-<i>α</i>-phosphatidylinositol-4-phosphate (PI(4)P; porcine brain), cholesterol (ovine wool) and 25-hydroxycholesterol (Avanti Polar Lipids, Alabaster, AL). The structures of the unlabeled phospholipids used in this project are shown in Figure 14. The volatile organic solvents (primarily chloroform) of the commercial lipid stocks were removed by a stream of nitrogen gas. The residual solvents were further dried in a high-vacuum evaporator for 1 – 2 h.

![Chemical structures of phospholipids](image)

**Figure 14:** Chemical structures of phospholipids. (A) DOPC and (B) PI(4)P were used in the DPI-based membrane binding assays.

For DPI-based assays, DOPC with varying mol% of PI(4)P, cholesterol and 25-hydroxycholesterol were reconstituted overnight at 4 °C in DPI
running buffer: 10 mM K$_2$HPO$_4$ and 137 mM NaCl (pH = 7.4). Large unilamellar vesicles (LUVs) were freshly prepared in glass vials immediately before experimentation by means of repeated extrusion through polycarbonate membrane (pore size = 100 nm; Avestin, Ottawa, ON) using a mini syringe-extruder (Avanti Polar Lipids, Alabaster, AL). Lipid samples were passed through the membrane for at least 15 – 20 times to ensure formation of uniform vesicles. The final lipid concentration of the extruded LUVs was 0.5 mM.

For FRET-based transfer assays, small unilamellar vesicles (SUVs) were prepared that contained DOPC with 3 mol% FRET-acceptor N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Alabaster, AL), commonly abbreviated as N-Rhod-PE. For some experiments, these vesicles also contained 4 mol% PI(4)P. Dried lipids were re-suspended overnight in DPI running buffer, sonicated using a probe sonicator at an amplitude of 30 % for 10 – 15 min until the suspension became homogeneous, and repeatedly extruded through membrane with pore size of 50 nm, as explained above. The SUVs were prepared at a concentration of 0.2 mM. The sizes of both type of vesicles were determined by a DynaPro-99-E-50 dynamic light scattering (DLS) spectrophotometer (Protein Solutions, Lakewood, NJ).

### 2.8. Dual polarization interferometry (DPI)

Purified OSBP, originally stored in IMAC elution buffer, was reconstituted in DPI running buffer using Econo-Pac® 10DG desalting gravity-flow columns (Bio-Rad Laboratories Canada, Mississauga, ON). Approximately 3 mL of protein sample was loaded on the column and eluted in 4 mL of DPI buffer.
Membrane-binding assays were performed to study the interaction of wtOSBP and its mutants with planar lipid bilayers containing DOPC with various ligands. The instrument used for this purpose, named AnaLight® Bio200 dual polarization interferometer (Farfield Group and Biolin Scientific, Stockholm, Sweden), was first equilibrated with DPI running buffer for 1 h. An unmodified silicon oxynitride sensor chip (AnaChip™ FB 80; Farfield Group and Biolin Scientific, Stockholm, Sweden) was then installed and the regions on the chip surface that provided clear pattern of interference fringes were selected for experimental observations. All reagents and buffers used during the assay were degassed for at least 30 min prior to use. The experiments were performed at 20 °C.

![Image](image.png)

**Figure 15: Illustration of events occurring on DPI sensor during a membrane-binding assay.** A simple depiction of vesicle immobilization and bilayer formation on the surface of a DPI sensor is shown here. The injected protein interacts with the bilayer and alters the refractive index of the medium, which is recorded in real-time as a phase shift of dual polarized light.

At first, the new chip was calibrated by injecting 80% ethanol and ultrapure water. This was followed by an injection of ~800 µL of desired LUVs with lipid concentration of 0.5 mM. The vesicles were allowed to immobilize and form bilayers on the chip surface. The flow rate was set to 25 µL/min and the injection of vesicles was performed for 8 min. Unilamellar vesicles are known to adsorb from the bulk solution onto the surface of solid support (in this case, the sensor chip) and spontaneously fuse with each other during the early part of the bilayer formation process where only vesicles
populate the surface. The fused vesicles begin to rupture at a critical surface coverage resulting in the co-existence of bilayer islands, intact vesicles and bare surface. Finally, a consistent planar supported lipid bilayer (SLB) is formed which covers the entire surface (Mashaghi et al., 2008) (Figure 15). SLB formation depends on the vesicular lipid composition and other factors that include charge, roughness, and cleanliness of the surface as well as pH, osmotic strength and ionic strength of the solution in which the vesicles are prepared (Castellana and Cremer, 2006).

After a stable bilayer was formed with a thickness of 4 – 5 nm, a quick injection of DPI buffer was performed (100 µL/min for 2 min) to remove excess lipids. Approximately 600 – 700 µL of required concentration of OSBP was then fed into the system at flow conditions identical to that used for lipid injection. The signal obtained from the DPI upon injecting vesicles and protein (Results, Section 3.2, Figure 20, Steps # 3 and 4), in the form of transverse magnetic (TM) and transverse electric (TE) phase shifts, was resolved and the specific mass of OSBP adsorbed/mm$^2$ of bilayer was calculated by AnaLight® Explorer software (Farfield Group and Biolin Scientific, Stockholm, Sweden). These values were plotted against time to obtain real-time association curves. To gain a better understanding of OSBP’s binding behaviour with membranes containing PI(4)P or sterols, the maximum specific mass of bound OSBP was also determined using GraphPad Prism® (ver. 6.0; GraphPad Software, La Jolla, CA) and plotted either as a function of mol% of ligand in the immobilized phospholipid bilayer or as a function of protein concentration, in order to obtain saturation binding curves.

At the end of protein injection, the surface-adsorbed lipid-protein layer was disintegrated by injecting 1 mL of each 2 % Hellmanex® cleaning concentrate (Hellma Canada Limited, Markham, ON) and 2 % SDS, successively, at a flow rate of 100 µL/min. The sensor was regenerated by
injecting 80 % ethanol at the same flow rate. A typical response obtained from the DPI instrument during an experiment is shown in the Results, Section 3.2 (Figure 20).

2.9. PI(4)P immunodetection assay

In order to test the hypothesis that wtOSBP extracted PI(4)P from phospholipid bilayers immobilized on the surface of the DPI sensor, it was necessary to detect PI(4)P in the protein fractions dissociating from these bilayers. To achieve this goal, wtOSBP, which interacted with bilayers containing either DOPC or DOPC + varying mol% of PI(4)P, was collected from the effluent flowing out of the DPI instrument. The protein sample was lyophilized in 1 mL fractions overnight at -80 °C. In order to dissolve PI(4)P, a 2:1:0.8 mixture of CH₃OH:CHCl₃:H₂O was added to the freeze-dried samples and vortexed extensively. After overnight incubation, the extracted PI(4)P was blotted on Amersham Hybond™ nitrocellulose membrane (GE Healthcare Canada, Mississauga, ON). The membrane was blocked with blocking buffer, probed with anti-PI(4)P mouse monoclonal IgM (1:500; Echelon Biosciences, Salt Lake City, UT) followed by goat anti-mouse IgM-HRP conjugate secondary antibody (1:1000; Santa Cruz Biotechnology, Dallas, TX), and visualized by adding TMB, as described in Section 2.6. Blots of lipid standards, containing 0.1, 1, 10 and 100 pmol of PI(4)P, were also assayed to provide a reference for comparison of signal intensities of experimental samples. DPI buffer, devoid of any protein, was injected over lipid bilayers and collected from the DPI effluent to serve as a negative control.

An alternate assay was developed to circumvent some technical difficulties with the method indicated above (see Results, Section 3.3.5, for details). In this, after performing an injection of wtOSBP (or DPI buffer as
negative control), the chip was removed from the instrument and PI(4)P-containing DOPC bilayer was solubilized overnight in a 2:1:0.8 mixture of CH$_3$OH:CHCl$_3$:H$_2$O. The samples were blotted and probed with anti-PI(4)P antibody, as explained above. The aim was to observe a loss-of-PI(4)P signal in lipids extracted from the bilayers that were exposed to wtOSBP, in comparison to the control. PI(4)P standards ranged from 10 nmol to 1 pmol.

2.10. Quantification of fluorescent lipid analogs

22-NBD-cholesterol (Setareh Biotech LLC, Eugene, OR) is a commercially available fluorescent analog of cholesterol that has a peak excitation wavelength ($\lambda_{\text{max}}$) of 469 nm. Serially diluted stocks of this compound were prepared in methanol and the absorption spectra were acquired by a UV spectrophotometer. The molar extinction coefficient of 22-NBD-cholesterol in methanol is 21,000 M$^{-1}$ cm$^{-1}$ (Johnson and Spence, 2010). Using this and the measured $A_{469}$ values, the concentration of the fluorescent sterol was determined by Beer’s Law (see eq. 1 in Section 2.4).

The fluorescent phospholipid analog N-Rhod-PE (Avanti Polar Lipids, Alabaster, AL) is known to have a peak excitation wavelength ($\lambda_{\text{max}}$) of 560 nm and a molar extinction coefficient of 75,000 M$^{-1}$ cm$^{-1}$ in methanol (Johnson and Spence, 2010). This compound was quantified by the same approach as outlined above for 22-NBD-cholesterol. The chemical structures of these lipid analogs are displayed in Figure 11.

2.11. Binding assay of 22-NBD-cholesterol

The ability of wtOSBP and OSBP-HH/AA to bind monomeric sterol was examined by means of 22-NBD-cholesterol binding assay. For this, each
protein was prepared in DPI buffer at a concentration of 200 nM and a total volume of 3 mL was aliquoted in a quartz cuvette (Hellma Canada Limited, Markham, ON). The sample was then titrated by serial additions of fixed (initially) or incremental (during later part of the assay) volumes of 22-NBD-cholesterol stocks (25 µM and 100 µM for this assay) prepared in methanol. After each addition, the protein-ligand mixture was allowed to equilibrate at room temperature for 10 min on a tube rotator. After exciting the fluorophore at 469 nm, fluorescence emission from NBD was scanned between 515 and 550 nm using PTI QuantaMaster™ QM-2001-4 spectrofluorometer (Horiba Canada Inc., London, ON). To prevent protein denaturation and loss of function, the total concentration of methanol was kept under 1 % (v/v).

Fluorescence intensity at the wavelength of maximum emission (λ_em), i.e. 537 nm, was recorded at each step and plotted against the total concentration of 22-NBD-cholesterol added, to obtain a binding curve. The data were normalized and fitted to a one-site specific binding model in GraphPad Prism® (ver. 6.0; GraphPad Software, La Jolla, CA) according to the following equation:

\[
Y = \frac{B_{\text{max}}X}{K_d + X}
\]  

(eq. 2)

In the above equation, Y denotes specific binding; \(B_{\text{max}}\) is the maximum specific binding (maximum in case of normalized data), in the same units as Y; X represents concentration of fluorescent ligand added to the protein sample; and \(K_d\) denotes equilibrium binding constant, in the same units as X. \(K_d\) values provided an insight into the affinity of the fluorescent cholesterol analog for OSBP and its mutant. The assay was performed with bovine serum albumin (BSA), in place of OSBP, as a control.
2.12. Competitive binding assays of 22-NBD-cholesterol

The ability of natural sterols to competitively replace 22-NBD-cholesterol from the binding pocket of wtOSBP and OSBP-HH/AA was examined. For this, 3 mL of 200 nM protein in DPI buffer was saturated with 500 nM 22-NBD-cholesterol (prepared in methanol). After 4 h of equilibration on a tube rotator at room temperature, the sample was titrated by serial additions of fixed (initially) or incremental (during later part of the assay) volumes of either cholesterol or 25-hydroxycholesterol stock (25 µM and 100 µM for this assay) prepared in isopropanol or ethanol, respectively. The remainder of the experiment was performed as outlined in Section 2.11. Fluorescence intensity was plotted as a function of concentration of competing ligand, in this case, cholesterol or 25-hydroxycholesterol, to obtain a competitive binding curve. The data were normalized and the ligand concentration was log-transformed to fit a one-site competitive binding model in GraphPad Prism® (ver. 6.0; GraphPad Software, La Jolla, CA) according to the following equation:

\[
Y = \min + \frac{\max - \min}{1 + 10^{(X - \log EC_{50})}}
\]  
(eq. 3)

In the above equation, Y denotes total binding; max and min represent maximum binding in the absence of unlabeled ligand and non-specific binding, respectively, in the same units as Y; X is the log concentration of unlabeled ligand added to the protein sample; and \( EC_{50} \) denotes the concentration of competitor that results in binding half-way between max and min, in the same units as X. \( EC_{50} \) values provided an insight into the efficacy of natural sterols in displacing 22-NBD-cholesterol from the binding pocket of OSBP. The assay was performed on bovine serum albumin (BSA) as a control.
2.13. OSBP-mediated sterol transfer by FRET

The ability of OSBP to transfer ORD-bound sterol ligand to an acceptor vesicle was examined by measuring the rate of Förster resonance energy transfer (FRET) between OSBP-bound 22-NBD-cholesterol and vesicle-resident N-Rhod-PE. SUVs used for this purpose contained DOPC and 3 mol% N-Rhod-PE. They were either devoid of PI(4)P (control) or harboured 4 mol% PI(4)P (experimental). At first, 4 µM protein (both wild-type and OSBP-HH/AA) was equilibrated with 0.8 µM 22-NBD-cholesterol for 30 min at room temperature on a rotator. The 5:1 protein:ligand ratio was chosen to allow complete binding of all ligand molecules to the protein prior to the transfer assay and to ensure high starting fluorescence count from 22-NBD-cholesterol. At the end of the incubation period, equal volumes of protein-ligand complex and 0.2 mM acceptor SUVs were mixed together in a quartz cuvette. The mixing was performed by a stop-flow apparatus attached to a PTI QuantaMaster™ QM-2001-4 spectrofluorometer (Horiba Canada Inc., London, ON). A 50-fold molar excess of SUVs, compared to protein, was used to ensure delivery of sufficient amounts of 22-NBD-cholesterol to the acceptor vesicle membranes. 22-NBD-cholesterol was excited at the wavelength of 469 nm. The decay in fluorescence emission was recorded at 535 nm while a gain in emission by N-Rhod-PE was recorded at 581 nm. The phenomenon was monitored for 360 s and the data from each assay were collected in triplicates.

The fluorescence intensities at both emission wavelengths were plotted as functions of time to obtain rate curves. Only the data of fluorescence gain from N-Rhod-PE were fitted to a two-phase exponential association model in GraphPad Prism® (ver. 6.0; GraphPad Software, La Jolla, CA) according to the following equation:

\[
Y = Y_0 + \text{Span}_1 \times (1 - e^{-k_1 t}) + \text{Span}_2 \times (1 - e^{-k_2 t})
\]

(eq. 4)
where, \[ \text{Span}_1 = (\text{Plateau} - Y_0) \times \text{Percent}_1 \times 0.1 \]

and \[ \text{Span}_2 = (\text{Plateau} - Y_0) \times (100 - \text{Percent}_1) \times 0.1 \]

In the above equations, \( t \) represents time in seconds; \( Y \) denotes gain in fluorescence by \( N \)-Rhod-PE; \( Y_0 \) denotes fluorescence intensity of \( N \)-Rhod-PE when time (\( t \)) is zero, in the same units as \( Y \); \( \text{Plateau} \) is the fluorescence intensity of \( N \)-Rhod-PE at equilibrium, in the same units as \( Y \); \( k_1 \) and \( k_2 \) are the rate constants of fast and slow phases, respectively, expressed in \( s^{-1} \); and \( \text{Percent}_1 \) represents the percentage of signal due to the fast phase. The slow phase was presumably caused by the spontaneous transfer of free 22-NBD-cholesterol to acceptor liposomes. Therefore, the rate constant of the fast phase (\( k_1 \)), that corresponded to the ligand transfer rate by OSBP, was considered for analysis.

The statistical significance of difference in the transfer rates (\( k_1 \)), in the presence and absence of PI(4)P in the acceptor membrane, was evaluated for each protein type, by performing an unpaired parametric Student’s \( t \)-test. The same analysis was performed to test the significance of difference in the \( \text{Plateau} \), in other words the maximum amount of sterol transferred to the acceptor vesicle, between wtOSBP and OSBP-HH/AA. The results of these comparisons were plotted as bar graphs.
Chapter 3

Results

This chapter details the observed trends and outcomes of the binding and transfer assays performed on the DPI and spectrofluorometer. The results provide a better comprehension of the membrane-binding preference of OSBP, based on the chemical nature of the constituent sterol and PIP ligands residing in the bilayers. My experiments highlight the role of PHD and ORD in enabling the wild-type protein to associate with specific type of membranes and transfer its sterol cargo. These studies were validated by the use of mutant versions of OSBP with impaired or altered domain functionalities. The parameters defining the kinetics of binding and transfer behaviour of OSBP have been reported. Lastly, the failed attempts to validate ligand extraction by OSBP’s have also been documented here along with the consequences of process improvisations and alternate analysis.
3.1. Expression and isolation of OSBP from Sf21 cells using Baculovirus Expression Vector System (BEVS)

3.1.1. Sf21 growth and infection with baculovirus

Sf21 displayed a healthy exponential growth as monolayers. Over several passages, the average cell density at the end of each generation was approximately $5 \times 10^6$ cells/mL with 90 – 95% viability (Figure 16A). Normal Sf21 cells displayed a spherical and somewhat granular morphology. Cells in a given population were unequal in sizes and attached firmly to the culture flask to form adherent layers. According to the literature, the mean diameter of Sf9 cells, a clonal isolate of Sf21 cells, is $18.5 \pm 1.5 \mu m$ (Gotoh et al., 2008).

At the end of the incubation period with baculovirus expression vectors carrying the desired coding sequences of human wtOSBP or its mutants, morphological differences between healthy proliferating cells and virus-infected cells became visually apparent. The diameter of cells increased considerably after infection and therefore, cell size alone could be used as a very good indicator of successful infection, not protein expression, as reported by many other groups (Sander and Harrysson, 2007; Radner et al., 2012; Üstün-Aytekin et al., 2014). The morphological features of healthy and infected cells are displayed in Figure 16B. Also, a decrease in the viability was observed in the infected population along with a marked decrease in the growth rate. For OSBP expression, MOI of 0.2 was used as a standard for infecting Sf21 cultures (see Section 3.1.3 for explanation).
Figure 16: Density and morphology of Sf21 cells in culture. (A) The total cellular density and % viability of Sf21 cultures maintained as adherent monolayers are shown. Data are mean ± S.E.M. of quadruplicate cell samples collected after each passage of cultures grown at different time-points. (B) Bright field images of Sf21 cells, grown as monolayers (upper panel) and suspension cultures (lower panel), are shown. The lower panel shows morphological differences between healthy vs. virus-infected cells. The images were captured by a digital camera attached to an inverted microscope using 40X objective 3 days after seeding (upper panel) and 3 days after infection (lower panel). Scale bar = 20 µm.
3.1.2. Extraction and purification of OSBP

Chromatographic isolation of His-tagged OSBP from baculovirus-infected cells was semi-automated by the FPLC system. During the process, all the undesired intracellular proteins expressed in virus-infected Sф21 cells were eliminated resulting in isolation, purification and enrichment of recombinant OSBP. This was evident from the chromatogram obtained from a typical run (Figure 17).

![Figure 17: OSBP purification by immobilized metal affinity chromatography](image)

Figure 17: OSBP purification by immobilized metal affinity chromatography. Chromatogram generated after a round of purification of OSBP by semi-automated IMAC-FPLC is shown. Clarified Sф21 cell lysate was equilibrated overnight with cobalt-iminodiacetic acid (Co^{2+}-IDA) resin at 4 °C. After packing the resin into a column, weak column binders were washed off and 6×His-tagged OSBP was isolated with phosphate buffered saline (pH = 7.4) containing 20 mM and 200 mM imidazole, respectively. Protein elution was monitored by recording absorbance of each eluted fraction at 280 nm.

The UV absorbance of the mobile phase at 280 nm decreased from an initial high value of 2.4 to a baseline value of 0.4 which denoted removal of the majority of proteins from the cell lysate. After a quick wash of the
stationary phase with a low concentration of imidazole, which eliminated other His-containing proteins bound weakly to the column, 6×His-OSBP elution was observed by a “peak” in the UV signal originating from the competitive displacement of the protein from Co²⁺-IDA by high concentration imidazole buffer.

### 3.1.3. Quantification and characterization of purified OSBP

Quantification of OSBP in the IMAC fractions by the Coomassie reagent showed that approximately 1.0 – 1.5 mg of OSBP was isolated per litre of Sf21 culture. A sample of wtOSBP that yielded a concentration of 2 µM by the Bradford method, was found to have a calculated concentration of 1.85 µM when the sample’s $A_{280}$ value was translated by the Beer’s law ([Figure 18, A and B](#)). The two estimates were reliably close to each other and a mean value was therefore considered for every experiment. The identity of wtOSBP in the fractions eluted from the IMAC column were confirmed using molecular weight analysis and immunodetection.

![Figure 18](#)

*Figure is continued on the next page...*
Figure 18: Quantification and characterization of purified OSBP. (A) An UV absorption spectrum of wtOSBP is shown with imidazole as a reference. (B) A BSA standard curve used for estimating OSBP concentration using Bradford protein quantification assay, is displayed here. Reported data are mean ± S.E.M. of quadruplicate trials for each sample. (C) Molecular weight analysis of wtOSBP using SDS-PAGE is shown in this image. Size-based separation of proteins from baculovirus-infected Sf21 cells (lanes 2 & 6), clarified Sf21 lysate (lanes 3 & 7), unbound fraction from IMAC column (lanes 4 & 8) and eluted protein (lanes 5 & 9) was performed on a 10% polyacrylamide gel. A comparison of wtOSBP yield is also provided between samples obtained from cells infected at an MOI of 0.1 for 72 h (lanes 2 – 5) vs. those infected at an MOI of 0.2 for 48 h (lanes 6 – 9). Molecular weight markers were loaded in lanes 1 and 10. (D) Immunodetection of wtOSBP by Western blot is shown here. Lane identities of samples probed with mouse anti-OSBP mAb (dil. 1:200) are the same as that of SDS-PAGE. Both 2D and 3D images of SDS-PAGE and Western blot were processed by Image Lab™ Software (ver. 5.2.1, Bio-Rad Laboratories Inc.).
SDS-PAGE results showed a distinct band, near the 100 kDa marker, in the sample containing pure OSBP (Figure 18C). At the same location, Western blot results showed the presence of OSBP by anti-OSBP mAb (Figure 18D). The predicted molecular mass of the protein was ~90 kDa. During the viral infection period, the expression level of the recombinant OSBP was monitored by SDS-PAGE of crude S/21 lysates collected at the end of each day of infection (Supplemental Figure 1). Viral transductions were initially carried out at an MOI of 0.1 and OSBP-expressing cells were harvested 72 h following infection. However, in order to augment protein yield, the MOI was changed to 0.2 and incubation period was decreased to 48 h. This modification resulted in an increase in the protein yield from ~0.5 mg/L to ~1.5 mg/L of culture volume, as evident from the lanes 5 vs. 9 of the SDS-PAGE/Western blot images (Figure 18, C and D).

3.2. Assessment of DPI sensitivity and optimization of experimental conditions

Before designing assays to analyze lipid-protein interactions using DPI, it was essential to test the sensitivity of the instrument in detecting changes in the collective mass of biomolecules deposited on the chip surface. Therefore, extruded vesicles containing either DOPC or DOPC with sterols (cholesterol and 25-hydroxycholesterol) and PI(4)P were prepared and subjected to size-determination by dynamic light scattering. According to the size-distribution, the majority of LUVs fell within a radius range of 60 – 100 nm in a given population of DOPC vesicles (Figure 19A). Data obtained from 20 acquisitions, of each type of vesicle population, indicated that the mean radius of the predominant LUV species was 92.8 nm for DOPC only, 88.2 nm for DOPC + 2 mol% PI(4)P, and 83.2 nm for DOPC + 10 mol% 25-hydroxycholesterol (Figure 19B). These values were within the
recommended size range of LUVs used for assays involving membrane immobilization on planar surfaces (Karst et al., 2012; Ouberai et al., 2013; Barroso et al., 2016).

Figure 19: **Size determination of large unilamellar phospholipid vesicles.** Percent distribution of LUVs based on their sizes (A) and mean sizes of predominant LUV species obtained from 20 acquisitions (B) in 0.5 mM samples of DOPC, DOPC + 2 mol% PI(4)P and DOPC + 10 mol% 25-HC Chol that were prepared in DPI buffer by repeated extrusion through polycarbonate membranes (pore size = 100 nm). Data were obtained from a dynamic light scattering spectrophotometer.

Real-time response from the DPI instrument during a typical membrane-binding assay has been shown in **Figure 20.** The output signal showed phase shifts in the transverse magnetic (TM) and transverse electric (TE) polarizations of the interfering beams of light during various stages of the experiment. The specific binding mass of OSBP (ng/mm²), depicted in the graphs presented in the subsequent sections, were obtained by resolving the signal obtained from the protein layer adsorbed on top the surface-immobilized lipid bilayer. It was found that the DPI technology was capable of detecting changes in the deposited mass of protein in the order of $10^{-1}$ ng/mm².
Figure 20: DPI response during a protein-bilayer association experiment. TM and TE phase shifts on the sensing channels of the DPI system during a typical bilayer binding assay is shown. The baseline response is obtained from a continuous flow of DPI buffer over the sensor surface. Various steps involved during an experimentation cycle are: 1) chip calibration with 80 % ethanol, 2) calibration of bulk density with ultrapure water, 3) adsorption of LUVs and subsequent formation of immobilized bilayers, 4) protein association with (and dissociation from) the bilayer, 5) bilayer removal by 2 % hellmanex / SDS and 6) chip regeneration with 80 % ethanol.
Figure 21: Profiles of bilayer immobilization on DPI sensor. Real-time adsorption, immobilization and rupture of LUVs composed of: A) DOPC only, B) DOPC + 2 mol% PI(4)P, C) DOPC + 5 mol% cholesterol, and D) DOPC + 5 mol% 25-hydroxycholesterol, followed by formation of stable bilayers on the DPI sensor have been depicted here. The concentration of phospholipid vesicles used for each trial is indicated in the legend. Reported data are mean thickness obtained from duplicate injections of each vesicle type.

Once the size of vesicles was established, the next step was to find the optimal concentration of LUVs that would form stable bilayers. LUVs made of DOPC only, DOPC + 2 mol% PI(4)P, DOPC + 5 mol% cholesterol, and DOPC + 5 mol% 25-hydroxycholesterol were injected over the DPI sensor at varying concentrations (0.08, 0.16, 0.32, 0.64, 1.27 and 2.54 mM). Adsorption profiles indicated that each type of vesicle was capable of forming SLBs of 4 –
5 nm thickness when injected at a concentration of 0.32 mM or higher (Figure 21, A – D). These measurements were in agreement with the thickness of SLBs reported by Reimhult and co-workers (Baumann et al., 2011). At lower concentrations (0.16 nM and 0.08 nM), vesicles containing only DOPC were unable to form coherent bilayers in spite of prolonged duration (10 min) of flow of vesicles. This was evident by the thickness of < 3 nm, measured as an average across the whole chip surface, which indicated incomplete surface coverage and possible presence of bilayer islands and bare spots on the biosensor. Also, the rate of formation of SLBs increased with the lipid concentration of injected vesicles in a dose-dependent manner. From the observed trend, 0.5 mM was chosen for the subsequent lipid-protein interaction assays.

3.3. Lipid recognition and membrane binding by OSBP

3.3.1. OSBP bound transiently to neutral DOPC bilayers

Since DOPC constituted the bulk of LUVs used for DPI experiments, it was important to study the interactions of OSBP with neutral bilayers, devoid of any sterol or PIP ligand, to establish a baseline binding response. Therefore, 1 µM protein was injected over a bilayer, made of DOPC only, at a rate of 25 µL/min while the interaction was recorded for 10 min and resolved as explained earlier (see Methods, Section 2.8). The profile of protein association, as a function of time, indicated substantially lower mass of protein deposited per unit area of surface-adsorbed bilayer in the case of wtOSBP as compared to OSBP-HH/AA (Figure 22). The maximum bound masses of the wild-type and the HH/AA mutant of the protein were ~0.5 ng/mm² and ~0.9 ng/mm² of DOPC bilayer surface, respectively.
After the commencement of protein injection, wtOSBP formed a temporary layer over the membrane surface that dissociated rapidly as soon as the injection ended, as indicated by the sudden drop in its specific mass to the baseline (Figure 22). In contrast, OSBP-HH/AA remained bound to the bilayer surface throughout the duration of flow and exhibited much slower dissociation after the cessation of flow. This showed that the wild-type protein has much lower affinity for neutral phospholipids, such as PC, and therefore, exhibits poorer membrane binding than its mutant counterpart.

**Figure 22: Interaction of OSBP with immobilized DOPC bilayer on DPI sensor.** A typical real-time mass association curve is shown that depicts the binding behaviour of wtOSBP and OSBP-HH/AA with bilayers composed only of DOPC. The termination of sample injection is indicated by the arrow. Reported data are representatives of duplicate trials of each sample.

Owing to the sensitive nature of the instrument in recording the slightest changes on the surface, minor day-to-day variation in the purity of protein sample as well as the surface properties of the sensor chip contributed to appreciable variance in the adsorbed mass data. However, every attempt was made to replicate as many trials as possible to quantify the variance.
3.3.2. *Binding affinity of OSBP was higher for cholesterol-containing membranes in comparison to membranes containing oxysterol.*

When sterols were incorporated in the membrane, the overall shape of the association curves for wtOSBP remained essentially similar to that of the neutral DOPC bilayers.

![Graph A](image1.png)  
**Figure 23:** Ligand-concentration dependent binding profiles of wtOSBP with immobilized DOPC bilayers containing sterols. Real-time associations of wtOSBP with DOPC bilayer containing varying mol% of 25-hydroxycholesterol (A) and cholesterol (B) are shown here. Reported data are means of triplicate trials of each sample.
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The maximum bound masses of the protein remained indistinguishable for DOPC bilayers containing 25-hydroxycholesterol when compared with the ones devoid of any recognizable ligand (Figure 23A). Interestingly, the response was markedly different for cholesterol-containing membranes. Approximately two-fold higher masses of wtOSBP bound to membranes containing cholesterol rather than the protein’s known higher-affinity ligand, 25-hydroxycholesterol (Figures 23B). As evident from the figure, the specific masses of adhering wtOSBP did not show any significant change when the content of either cholesterol or 25-hydroxycholesterol in the membrane was varied over the following range: 0, 2, 5, 10 and 20 mol%.

3.3.3. Compared to sterols, OSBP showed slower dissociation from PI(4)P-containing membranes.

The binding of wtOSBP to PI(4)P-containing membranes had dramatic consequences. At the onset of injection, the wild-type protein adsorbed quickly to the bilayer followed by a gradual slow desorption over the course of sample flow. Moreover, with increasing mol% of PI(4)P in the membranes, the shape of the curves reflected distinct association. WtOSBP took longer time to saturate the bilayers with each increment in PI(4)P concentration (Range: 0, 2, 4, 6 and 8 mol%), as indicated by the dotted lines in Figure 24.

The behaviour of wtOSBP binding to PI(4)P-containing bilayers was markedly different from those containing sterols for various aspects of the binding profiles. For example, the characteristic shape of protein-PI(4)P association curves was distinctly different from the relatively “flat” protein-sterol association curves. When compared to sterol-containing bilayers, the dissociation of wtOSBP from PI(4)P-containing membranes was much slower at higher PI(4)P concentration, such as 6 and 8 mol%. This was indicated by a DPI signal that almost never came back to baseline at the termination of
protein injection, thereby indicating incomplete desorption of the interacting protein layer (Figure 24).

![Specific Mass vs Time](image)

**Figure 24:** Ligand-concentration dependent binding profiles of wtOSBP with immobilized DOPC bilayers containing PI(4)P. Real-time associations of wtOSBP to DOPC bilayer containing varying mol% of PI(4)P are shown here. The dotted vertical lines denote the time-points when maximum mass of wtOSBP adsorbed on bilayers with corresponding mol% of PI(4)P. Reported data are means of triplicate trials of each sample.

Unlike either cholesterol or 25-hydroxycholesterol, the maximum bound mass of protein increased with increasing mol% of PI(4)P in the bilayer. In spite of being consistent at lower ligand concentrations, membrane binding of wtOSBP, when PI(4)P was present, deviated significantly from that where sterols were present at higher mol% (Figure 24). At the highest tested concentration of ligand, i.e. 8 mol% of PI(4)P and 20 mol% of either sterol, the maximum specific mass of wtOSBP for bilayers with PI(4)P was approximately three-fold and six-fold higher than those with cholesterol and 25-hydroxycholesterol, respectively. The specific mass-to-ligand concentration curve for PI(4)P roughly followed a sigmoid-pattern showing membrane saturation with protein at 6 mol% of PI(4)P. In the case of sterols, however,
the graphs did not show a tendency towards increasing protein adsorption with increasing amount of sterol in the membrane (Figure 25).

Figure 25: Ligand-wise comparison of the membrane-binding behaviour of wtOSBP. Maximum specific masses of 1 µM wtOSBP bound to surface-adsorbed DOPC bilayers are shown as a function of varying mol% of ligands, namely, PI(4)P, cholesterol and 25-hydroxycholesterol. Reported data are means ± S.E.M. of triplicate trials.

3.3.4. OSBP associated in a dose-dependent manner with membranes containing PI(4)P, not sterols.

When binding assays were performed by maintaining a constant amount of ligand in the adsorbed membrane and varying the concentrations of interacting protein, wtOSBP associated in a dose-dependent manner only with bilayers containing PI(4)P, not sterols (Figure 26, A – C). The amounts of ligand, in vesicles, chosen for this test were 5 mol% of either sterol and 2 mol% of PI(4)P. While the shape of the binding curve of each dosage of wtOSBP remained more-or-less “flat” for both sterols, protein association with PI(4)P-containing membranes followed the characteristic pattern observed earlier (Section 3.3.3).
Figure is continued on the next page...
Figure 26: Dose-response of wtOSBP to immobilized DOPC bilayers. Real-time associations of varying concentrations of wtOSBP to DOPC bilayers containing (A) 5 mol% cholesterol, (B) 5 mol% 25-hydroxycholesterol, and (C) 2 mol% PI(4)P. The dotted vertical lines in curve C denote the time-points when the maximum mass of protein, at each corresponding dosage, adsorbed to the bilayer. Also shown is a plot of maximum specific masses of the protein (D) bound to bilayers containing 2 mol% of PI(4)P (red) and 5 mol% of either sterol (blue) are shown as a function of protein concentration. Reported data are means ± S.E.M of duplicate trials.

At the highest tested dose of wtOSBP (i.e. 2 µM), 1.0 ng of protein bound per mm² surface of adsorbed membranes containing PI(4)P while only 0.5 – 0.6 ng of protein bound to membranes containing sterols, resulting in the saturation of bilayer surface by protein. This is apparent from the binding saturation curve (Figure 26D). It is interesting to note that the rate of the initial rapid buildup of wtOSBP on the PI(4)P-harbouring bilayer increased with increasing concentration of injected protein sample. In addition to this, the time taken to reach the maximum bound mass was also observed to vary inversely with protein dosage, as indicated by the dotted lines in Figure 26C.
3.3.5. Immunodetection-based confirmation of PI(4)P-extraction from adsorbed membrane by wtOSBP remained inconclusive.

The association (and dissociation) profiles of OSBP with PI(4)P-bearing membranes hinted towards the possibility of ligand extraction from the bilayers by the wild-type protein (see Discussion and Outlook, for explanation). In order to test this hypothesis, immunodetection of PI(4)P extracted by wtOSBP was carried out by performing a fat-blot assay. The injected wtOSBP, that had flowed over and interacted with PI(4)P-bearing DOPC bilayers, was collected from the DPI effluent and was tested for the presence of PI(4)P using anti-PI(4)P mAb. However, the results proved inconclusive due to technical limitations. At first, the intensity of the blot signals looked directly proportional to the mol% of PI(4)P (Figure 27A, +wtOSBP), which explained why the protein remained bound for a longer duration with each increment in the ligand concentration in the bilayer. Interestingly, the developed colour was less intense for 6 mol% PI(4)P which was also in agreement with the shape of the corresponding association curve (Figure 24). In this specific instance, the protein remained bound to the membrane for a much longer duration after the termination of the injection as the DPI signal never returned to the baseline.

However, these results became perplexing when a similar trend was also observed for a control experiment that used only DPI running buffer instead of wtOSBP (Figure 27A, -wtOSBP). In fact, the control blots showed more intense signals which indicated that more PI(4)P was extracted by the buffer than the actual protein. This was extremely unlikely to happen. Moreover, when compared to the signals from PI(4)P standards (Figure 27B), the blots in Figure 27A showed much higher intensity while the maximum amount of PI(4)P available for extraction from the adsorbed bilayers with 6 mol% of PI(4)P was only about 4 pmol. Due to these discrepancies, the results were deemed inaccurate and have been ignored.
Figure 27: Immunodetection of PI(4)P. A) Fat blots probed with anti-PI(4)P mAb are shown for detection of PI(4)P in the DPI effluent collected after injecting 1 µM wtOSBP over bilayers containing varying amounts of PI(4)P. B) Fat blots depict absence of signal for wtOSBP injected over neutral DOPC bilayers and increased signal intensity for 2 µM protein, compared to 1 µM, when injected over DOPC + 2 mol% PI(4)P. Signals for PI(4)P standards (with indicated amounts blotted on the membrane) are also displayed here. C) Shown here is a failed attempt to detect PI(4)P extraction by wtOSBP by means of loss-of-PI(4)P signal from surface adsorbed bilayers after binding and dissociation of wtOSBP. The extractable PI(4)P amounts were found to be below the detection limit of this assay.

A second attempt was made to bypass the problems faced previously and obtain discernable results from the fat blot assay. This time, the aim was to detect the depletion of PI(4)P from the surface-adsorbed membrane after the completion of a wtOSBP injection, by dissolving the bilayer, probing the blots using anti-PI(4)P mAb and recording the loss-of-signal compared to buffer-injected controls. However, progress was hindered due to lack of signal at any mol% of PI(4)P for buffer-injected controls (Figure 27C). A quick look
at the PI(4)P standards made it clear that the assay was simply not sensitive enough to detect amounts of PI(4)P less than 10 pmol. Therefore, this approach was discarded altogether and the OSBP-HH/AA mutant was used to confirm the PI(4)P-extraction phenomenon.

3.3.6. OSBP extracted PI(4)P from surface-adsorbed bilayers after recognizing the ligand by its PHD.

Membrane binding assays were performed using the OSBP-RR/EE mutant, incapable of recognizing PIPs, at a single concentration of protein and ligand. The DPI response from an injection of 2 µM OSBP-RR/EE mutant over an adsorbed bilipid membrane with 2 mol% PI(4)P showed a complete absence of the characteristic initial buildup of mass and subsequent dissociation as seen in the case of wtOSBP (Figure 28A). The mutant associated with the bilayer in a fashion akin to the wild-type protein on neutral DOPC bilayer. This unequivocally showed that PHD is essential for recognition of PIP and docking of OSBP to the membrane for ligand extraction.

Figure is continued on the next page...
Figure 28: Association of OSBP mutants with bilayers containing PI(4)P. (A) DPI responses, depicting the real-time interaction of wtOSBP vs OSBPPRR/EE mutant with bilayers containing 2 mol% PI(4)P. (B) Association curves of OSBP-HH/AA with bilayers containing varying mol% of PI(4)P. (C) Maximum specific masses of the bound HH/AA mutant (vs. wtOSBP) as a function of PI(4)P density in the bilayers. Reported data are means ± S.E.M. of triplicate trials.

A second mutant, called OSBP-HH/AA, was tested to provide evidence in favor of PI(4)P-extraction from membrane by wtOSBP. OSBP-HH/AA was capable of recognizing PI(4)P in the adsorbed bilayers and therefore bound at high specific masses (**Figure 28B**). Judging by the essentially “flat” shape of
the curves coupled with very slow dissociation of protein from the membrane after the end of its flow, it could be said that this mutant was incapable of extracting PI(4)P from the membrane, as mentioned earlier by Ridgway and co-workers (Goto et al., 2016). The bound mass of OSBP-HH/AA increased with increasing mol% of PI(4)P ultimately reaching saturation at PI(4)P amounts in the bilayer beyond 2 mol% (Figure 28C). Unlike the wild-type protein, the time to reach maximum bound mass remained unchanged for trials with various concentrations of membrane PI(4)P and so were the association and dissociation rates.

3.4. Kinetics of sterol transfer by OSBP

3.4.1. 22-NBD-cholesterol showed similar binding affinity for wtOSBP and BSA.

A fluorescent cholesterol analog, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3ß-ol or 22-NBD-cholesterol, was used to study the binding of monomeric sterol with wtOSBP and OSBP-HH/AA. A study of the absorption behaviour of this fluorescent ligand at various solvent dilutions showed that it absorbed most light in the visible region of the electromagnetic spectrum with a maximum at the wavelength (λ<sub>max</sub>) of 469 nm. Maximum emission was recorded at the wavelength (λ<sub>em</sub>) of 535 nm.

The initial binding response of 22-NBD-cholesterol obtained by monitoring the emitted fluorescence intensity showed similar affinity of the ligand for both the wild-type and mutant forms of OSBP. At lower concentrations, the ligand bound incrementally to either protein in aqueous solution as seen in Figure 29, before saturating the binding sites on the proteins at concentrations above 150 nM. Although the observed maximal
binding, $B_{\text{max}}$, could not be quantified and compared between the proteins, normalizing the recorded fluorescence intensities helped in computing the dissociation constants. The estimated $K_d$ values for 22-NBD-cholesterol to wtOSBP and OSBP-HH/AA were $37.3 \pm 5.1$ nM and $34.0 \pm 2.6$ nM, respectively. Although these were not so different from each other, a control experiment with bovine serum albumin (BSA) produced a $K_d$ value of $20.0 \pm 6.0$ nM, which was surprisingly at the same ballpark as the values obtained for OSBP. This result hinted towards non-specific binding of 22-NBD-cholesterol with the wild-type and mutant forms of OSBP.

![Normalized Fluorescence Intensity vs. [22-NBD-Cholesterol] (nM)](image)

**Figure 29: Saturation binding curves for fluorophore-labeled cholesterol.** Total binding of monomeric 22-NBD-cholesterol to OSBP (wild-type and HH/AA mutant) and BSA (control) is shown here. The fluorescent ligand was added incrementally to 200 nM of specified protein every 10 min at room temperature. Fluorescence intensity recorded at each concentration of ligand is plotted on a linear scale and fitted to a one-site binding equation using a non-linear regression method. Reported data are as means ± S.E.M. of triplicate trials.
3.4.2. Natural sterols competed with 22-NBD-cholesterol for binding OSBP, but not BSA.

Specificity of 22-NBD-cholesterol for OSBP was examined by allowing natural sterols, such as cholesterol or 25-hydroxycholesterol, to compete with protein saturated with the fluorescent sterol analog. A steady decay in NBD fluorescence due to incremental addition of either natural sterol showed competitive displacement of the ligand from the sterol-binding pocket of OSBP (Figure 30, A and B). 25HC proved to be the most potent competitor requiring lower concentrations to reach the minimum fluorescence. This was evident by complete displacement of 22-NBD-cholesterol at 100 nM for both wtOSBP and OSBP-HH/AA. For cholesterol, a higher concentration was required to outcompete the fluorescent sterol, since the decay in fluorescence became exceedingly slow only after 150 – 200 nM of added cholesterol. Interestingly, no competition was observed in case of BSA (Figure 30C), which was an indication that 22-NBD-cholesterol bound BSA in a non-specific manner that was due to the absence of a conserved sterol-binding pocket. The competitive binding curves for wtOSBP and OSBP-HH/AA with log-transformed ligand concentration (Figure 30, D and E) enabled estimation of $EC_{50}$ values of cholesterol and 25-hydroxycholesterol for both proteins. These values are summarized in Table 1.

![Figure 30](image-url)
Figure 30: Competition binding curves for fluorophore-labeled cholesterol. Competitive displacement of 22-NBD-cholesterol from ligand-saturated (A) wtOSBP (B) OSBP-HH/AA, and (C) BSA by cholesterol and 25-hydroxycholesterol is shown. 500 nM of the fluorescent ligand was incubated with 200 nM of protein followed by incremental addition of natural sterols every 10 min at room temperature. Competition binding curves are plotted on a linear scale. Data from (A), (B) and (C) were normalized, re-plotted against log concentration (X-axis) and fit to one-site competition equation using a non-linear regression method. Behaviour of both sterols on wtOSBP vs. BSA (D) have been compared with that of OSBP-HH/AA vs. BSA (E). The estimated \( EC_{50} \) values of wtOSBP are reported in Table 1. Reported data are means ± S.E.M. of triplicate trials.

It should be noted that although the starting fluorescence of 22-NBD-cholesterol-bound OSBP (both wild-type and mutant) was recorded prior to the addition of either unlabeled sterol during the competition assays, the data point could not be plotted on the normalized data (Figure 30, D and E).
due to log transformation of the concentration of unlabeled sterol on the X-axis. The sigmoid-shape of the competitive binding of natural sterols to OSBP could not be appreciated due to lack of initial additions of minuscule concentrations of natural sterols in sub-nanomolar range. Nevertheless, the one-site competitive-binding model was able to predict the starting fluorescence and estimate the $EC_{50}$ values, tabulated in Table 1.

**Table 1: $EC_{50}$ values from competition assay.** Half-maximal effective concentrations ($EC_{50}$) of natural sterols for competitive binding to OSBP against 22-NBD-cholesterol.

<table>
<thead>
<tr>
<th>OSBP variant</th>
<th>$EC_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Wild-type</td>
<td>15.6 ± 0.7</td>
</tr>
<tr>
<td>HH/AA mutant</td>
<td>13.8 ± 0.7</td>
</tr>
</tbody>
</table>

3.4.3. **Presence of PI(4)P in acceptor vesicles did not alter the sterol-delivery rate of OSBP in FRET-based transfer assay**

A fluorescent phospholipid analog, $N$-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, commonly known as $N$-Rhod-PE, was used as a FRET partner of 22-NBD-cholesterol to study the transport of sterol to small unilamellar vesicles by wtOSBP and OSBP-HH/AA. This fluorescent probe showed light absorbance in the visible region of the electromagnetic spectrum with a $\lambda_{\text{max}}$ of 560 nm. For the experiment, small vesicles containing either DOPC and 3 mol% $N$-Rhod-PE or DOPC, 3 mol% $N$-Rhod-PE and 4 mol% PI(4)P were prepared and subjected to size-determination by dynamic light scattering. In a given population of these SUVs, the radius of the majority of the vesicles was found to be within 30 –
60 nm (Figure 31A). Data obtained from 20 acquisitions, indicated that the mean radius of the predominant SUV species was 57.5 nm for both types of DOPC vesicles, with or without PI(4)P (Figure 31B).

![Figure 31](image)

**Figure 31:** Size determination of small unilamellar phospholipid vesicles. (A) Percent distribution of SUVs based on their sizes and (B) mean sizes of predominant SUV species obtained from 20 acquisitions in 0.2 mM samples of DOPC + 3 mol% N-Rhod-PE and DOPC + 3 mol% N-Rhod-PE + 10 mol% PI(4)P prepared in DPI buffer by sonication and repeated extrusion through polycarbonate membranes (pore size = 50 nm). Data were obtained from a dynamic light scattering spectrophotometer.

Due to FRET, the NBD fluorescence was expected to be quenched over time with a concomitant increase in Rhodamine fluorescence, depending upon the rate of delivery of 22-NBD-cholesterol to acceptor vesicles by OSBP. It was evident from the rate curves (Figure 32, B and D) that the decrease in NBD fluorescence was substantially lower in magnitude when compared to the gain in Rhodamine fluorescence. The kinetic model representing two-phase exponential association was fitted to Rhodamine data to estimate the transfer rates. Only the rates of initial “fast” phases were considered in the analysis because they truly represented protein-mediated sterol transfer. The “slow” phases were caused by the spontaneous transfer of free fluorescent sterols to the acceptor membranes. For wtOSBP, a gradual increase in
transfer was observed which did not reach equilibrium during the period when Rhodamine fluorescence was monitored (Figure 32B). This was true for acceptor vesicles with or without PI(4)P. The differences in the estimated maximum fluorescence intensity at equilibrium for these vesicles were found to be statistically non-significant (Figure 32E, wtOSBP). The same observation was made for the rate constants, which did not change between the two types of vesicles (Figure 32C, wtOSBP).

Figure is continued on the next page...
Figure 32: Transfer of 22-NBD-cholesterol to small unilamellar vesicles by OSBP. (A) A general scheme to monitor sterol transfer by OSBP to acceptor vesicles, using FRET, is depicted. Also shown are rate curves depicting change in Rhodamine fluorescence as an indicator of sterol transfer by (B) wtOSBP and (D) OSBP-HH/AA. The insets depict change in NBD fluorescence for the corresponding assays. Rhodamine data were fitted to two-phase exponential association model using a non-linear regression method. The estimated rate constants of fast phases (C) and maximum intensity of Rhodamine fluorescence at equilibrium (E) were compared between wtOSBP and OSBP-HH/AA and displayed as bar graphs (F.I. = fluorescence intensity). Data are means ± S.E.M. of triplicate trials. Statistical significance of difference in these kinetic parameters between the two proteins and the two types of acceptor vesicles are indicated above each set of bars (unpaired Student t-test, * denotes p < 0.05, ns = non-significant).

For OSBP-HH/AA, the transfer rates were similar in magnitude when compared to wtOSBP and the rate constants for acceptor vesicles devoid of PI(4)P were not significantly different from the ones for vesicles containing PI(4)P (Figure 32C, OSBP-HH/AA). The rate curves indicated that the mutant, like the wild-type protein, did not reach an equilibrium within the observed duration of the assay (Figure 32D). Although the estimated maximum fluorescence intensity at equilibrium didn’t differ with the amount of PI(4)P in the acceptor vesicle, they were found to be significantly smaller than the values obtained from sterol transfer by wtOSBP indicating higher net delivery of sterols by the wild-type protein than the mutant (Figure 32E).
Chapter 4

DISCUSSION AND OUTLOOK

This section dissects the membrane binding behaviour of OSBP and its mutants based on the findings from the assays demonstrated on the DPI. Special emphasis has been given to the interaction of OSBP with membranes containing PI(4)P and the ability of this ligand to modulate the protein’s affinity for the membrane. The reasoning behind the kinetic rates, witnessed during the transfer of fluorescent sterol analogs to acceptor vesicles by OSBP, has been provided here. Also included is the description of the probable causes of experimental failures and justification of modified approaches along with suggestions of investigative designs for future. Taken together, this chapter illustrates my attempt to link the results with the characteristics already attributed to OSBP in the literature while highlighting some newfound trends.
4.1. Estimation of the maximum theoretical specific mass of membrane-bound OSBP

The DPI was operated under flow conditions and the adsorbed protein layers were arguably in a pseudo-equilibrium state. In other words, OSBP was associating with and dissociating from the immobilized phospholipid bilayer throughout the duration of its flow over the sensor chip. It was crucial to have a clear knowledge of how much surface of the bilayer could actually be occupied by OSBP, before drawing any meaningful conclusion from the binding isotherms obtained from the instrument. Therefore, a theoretical estimation of the expected DPI response due to complete coverage of the adsorbed membrane by the protein was pertinent.

In order to achieve that, the diameter of the sterol binding face of the OSBP-related domain (ORD) was calculated from the crystal structure of Osh4 (PDB ID: 1ZHX). It was found to be ~60 Å (Schulz et al., 2009), thus yielding an approximate circular surface area of 2830 Å². Therefore, the sterol-binding ORD of each OSBP molecule would interact with the adsorbed bilayer over an area of 2830 Å². One square millimeter of membrane surface was found to be occupied by approximately 3.54 × 10¹⁰ OSBP molecules (58.72 fmol) of OSBP, which was equivalent to 5.28 ng of OSBP/mm² (M.W. of OSBP = 90,000 g.mol⁻¹). If one had to assume that a DOPC bilayer covered the entire surface of the sensor chip, then based on the calculations outlined above, a specific protein mass of 5.28 ng/mm² would indicate 100 % surface occupancy by OSBP. For membranes containing PI(4)P, the number of PI(4)P molecules per unit surface area was calculated by dividing 1 mm² by 72.4 Å² (the approximate area of a phosphatidylcholine headgroup) (Lewis and Engelman, 1983; Kučerka et al., 2005) and multiplying the calculated value by the mol% of PI(4)P in the bilayers. If OSBP interacted with PI(4)P in 1:1 stoichiometry, then the maximum mass of protein that associates with the
membrane would be equivalent to the number of PI(4)P molecules on the upper leaflet of the adsorbed bilayer, until that number exceeds $3.54 \times 10^{10}$. The calculated maximum specific masses are shown in Table 2. The deviation of the observed specific masses from the ones calculated here are highlighted in Section 4.5.

**Table 2: Theoretical estimation of maximum mass of adsorbed protein on immobilized bilayers.** Maximum masses of protein theoretically expected to bind per mm$^2$ of immobilized bilayers containing varying mol% of PI(4)P in the DPIP-based assays. Masses have been calculated assuming 100% chip coverage by the bilayers and OSBP interaction with PI(4)P at 1:1 stoichiometry.

<table>
<thead>
<tr>
<th>Amount of PI(4)P in bilayer</th>
<th>Number of PI(4)P molecules/mm$^2$</th>
<th>Number of OSBP molecules/mm$^2$</th>
<th>Max. specific mass (ng/mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mol%</td>
<td>$1.38 \times 10^{10}$</td>
<td>$1.38 \times 10^{10}$</td>
<td>2.06</td>
</tr>
<tr>
<td>2 mol%</td>
<td>$2.76 \times 10^{10}$</td>
<td>$2.76 \times 10^{10}$</td>
<td>4.13</td>
</tr>
<tr>
<td>4 mol%</td>
<td>$5.52 \times 10^{10}$</td>
<td>* $3.54 \times 10^{10}$</td>
<td>* 5.28</td>
</tr>
<tr>
<td>6 mol%</td>
<td>$8.29 \times 10^{10}$</td>
<td>* $3.54 \times 10^{10}$</td>
<td>* 5.28</td>
</tr>
<tr>
<td>8 mol%</td>
<td>$1.10 \times 10^{11}$</td>
<td>* $3.54 \times 10^{10}$</td>
<td>* 5.28</td>
</tr>
</tbody>
</table>

* Under these circumstances, more PI(4)P molecules were available than the maximum number of OSBP molecules that could occupy the surface. Thus, the concentration of injected protein became the limiting factor for binding.

Similar estimations were not performed for bilayers containing sterols because the characteristic association followed by dissociation, as observed during the exposure of wtOSBP to PI(4)P-containing membranes, was absent for bilayers with sterols. Therefore, the specificity of protein-membrane interaction in those cases remains inconclusive and requires further investigation.
4.2. Behaviour of OSBP towards neutral PC bilayers

When compared to α-tocopherol transfer protein (TTP), another LTP studied earlier in our lab (Baptist et al., 2015), the interaction of wtOSBP with DOPC-only bilayers was not drastically different. TTP binds well to DOPC forming an adsorbed protein mass of ~0.75 ng/mm$^2$ when 1.0 µM of protein is exposed to an immobilized bilayer. In contrast, wtOSBP showed poorer binding, with an adsorbed mass of 0.5 ± 0.4 ng/mm$^2$. However, unlike wtOSBP, which was injected over a pure DOPC membrane, the TTP binding assay was performed on a bilayer made of 9:1 DOPC:DOPS. It should be noted that the “background” of a non-specific signal, caused by a change in the refractive index of the bulk medium, for any protein injected across the chip was ~0.2 ng/mm$^2$. It can be argued that in the absence of a charged headgroup, OSBP is not likely to associate with any phospholipid membrane, which agrees with the results obtained here. The reason behind this is that the protein does not have any specialized domain for recognizing or binding PC. Therefore, any change in the refractive index caused by wtOSBP was due to non-specific interactions of its solvent-exposed amino acids with DOPC headgroup, possibly explaining why the protein layer was unstable and dissociated immediately after the cessation of protein flow (see Results, Section 3.3.1, Figure 22). This was, however, not the case with OSBP-HH/AA, which formed a layer with about two-fold higher mass than wtOSBP, and dissociated at a much slower rate when the protein injection ended.

The His-524 and His-525 pair stabilizes the headgroup of PI(4)P at the entrance to the hydrophobic tunnel of ORD (Figure 4C and 4D) while the acyl chains remain inserted inside the pocket via non-specific interactions (Tong et al., 2016). His → Ala mutation in the ORD of OSBP-HH/AA renders the protein non-functional in terms of its ability to extract PI(4)P from membranes (Goto et al., 2016). Im and co-workers have elaborated that in the absence of ligand, potential phospholipid binding sites of Osh4 are exposed
and the protein is positioned for membrane docking (Im et al., 2005). In light of this knowledge, it can be said that the higher preference of OSBP-HH/AA for DOPC membranes than the wild-type protein was probably an outcome of the reduced polarity (increased hydrophobicity) of the mutant at the PI(4)P-accommodating site in the ORD. However, a lid-truncated structure of Osh4 (PDB ID: 1ZI7) shows that the two His residues are located far from the membrane-interacting surface of the protein (Figure 33). This weakens the above argument. Therefore, the precise reason for the lower adsorbed mass of wtOSBP to DOPC bilayers, compared to OSBP-HH/AA, remains unclear and warrants further investigation. It is possible that the mutant protein orients differently around the surface of bilayer which can alter its affinity for the membrane.

Figure 33: Position of the conserved His pair of Osh4 with respect to the surface of the bound membrane. X-ray crystallographic structure of Osh4/Kes1 with truncated N-terminal lid is shown here. The His-143 and His-144 pair (magenta) is crucial for stabilizing the headgroup of PI(4)P in the ORD. The β-barrel (a.a. 117 – 307), forming the hydrophobic pocket for harbouring ligand, is coloured yellow. The surface of the phospholipid membrane in contact with protein is coloured blue. The image is prepared in PyMOL (ver. 1.7.4.5) from the PDB structure 1ZI7 solved by X-ray diffraction at 2.5 Å resolution (Im et al., 2005).
4.3. Binding of OSBP to sterol-containing membranes

Binding of wtOSBP to sterol-containing bilayers showed some interesting trends. It is well known that 25-hydroxycholesterol displays higher affinity for wtOSBP than cholesterol, as evident from the $K_d$ of 10 nM of the former when compared to 170 nM of the latter (Dawson et al., 1989a; Ridgway et al., 1992; Wang et al., 2002, 2008). This attribute of the protein, however, was not reflected from its membrane binding behavior in this study. In other words, the DPI association curves, obtained from the experiments described in this thesis, showed nearly two-fold higher specific bound mass of protein over cholesterol-containing membranes than the ones with the same density of 25-hydroxycholesterol (see Results, Section 3.3.2, Figure 23) even though 25HC is a known high-affinity ligand of ORPs (Dawson et al., 1989a, 1989b).

Also noteworthy was the protein’s lack of preference for binding bilayers with 25HC, when compared to neutral DOPC bilayers. This was apparent from the similar magnitude of bound mass of protein to both types of membranes. It seems that the presence of either cholesterol or oxysterol changes the nature of the membrane and the protein, capable of sensing such a change, may very well adapt its affinity towards the membrane. However, the precise mechanism could not be deduced from the results in hand. Among the factors that regulate the protein binding, the membrane curvature could play a significant role. The lid region of yeast ORP Osh4 corresponds to an unstructured sequence, commonly known as the ALPS motif (Drin et al., 2007). Owing to the affinity of this domain for membranes with loose lipid packing, Osh4 was shown to bind preferentially to anionic membranes with high positive curvatures (de Saint-Jean et al., 2011). That being said, a tighter lipid packing, and to some extent formation of ordered lipid microdomains, can be expected due to incorporation of either oxysterol or cholesterol in the bilayers (Xu and London, 2000). Immobilization and
rupture of vesicles on the biosensor chip, to form planar surface-adsorbed bilayers, causes loss of positive membrane curvature. Taken together, these factors could be responsible for making the movement of sterol from the membrane to the ORD of OSBP energetically unfavorable on the DPI platform.

Both types of DPI assays, one in which the mol% of sterol was kept constant while the protein dosage was varied and the other in which the protein concentration was kept constant but the mol% of sterol in the membrane was varied, yielded somewhat similar responses. Although the shapes of the curves were distinguishable between the various protein doses, the maximum bound mass appeared to be fairly constant for the oxysterol. Cholesterol, on the other hand, produced similar-looking curves with the peak bound mass being constant throughout both assay types (see Results, Section 3.3.4, Figure 25A, -B and -D). Due to time constraints and lack of a steady supply of protein, the sterol-binding assays could not be performed with OSBP-HH/AA. In fact, the mutations in the His-524 and His-525 pair in the ORD is only known to hamper the extraction and transport of PIPs, not sterols (Im et al., 2005; de Saint-Jean et al., 2011). Therefore, the DPI response for this mutant is not expected to be markedly distinguishable from that of the wild-type. Nevertheless, this study should provide a decent control for the behaviour of OSBP towards sterol-containing membranes in the future.

4.4. High affinity of OSBP for membranes with PI(4)P

The response of OSBP towards PIP-bearing membranes provided useful insights into the events occurring at the OSBP-membrane contact sites. I chose to work with PI(4)P, a cognate ligand for the conserved PHD, found in several members of the ORP-family including the archetype OSBP.
After the discovery of a PIP binding site in the hydrophobic pocket of the ORD of yeast protein Osh4 (de Saint-Jean et al., 2011), Antonny and coworkers have elaborated that PI(4)P serves as an exchangeable ligand for sterol in the OSBP signaling pathway (Mesmin et al., 2013). The wild-type protein was able to station itself at the PI(4)P-containing bilayers on the DPI sensor, which was evident by a rapid increase in adsorbed protein mass during the early part of protein injection (see Results, Section 3.3.3, Figure 24). The recognition of PIP and subsequent docking of wtOSBP to PI(4)P-containing bilayers occurred by the virtue of the protein’s PHD. A double-arginine mutant of this domain, named OSBP-RR/EE, failed to identify PI(4)P and exhibited protein adsorption response similar to binding DOPC bilayers (see Results, Section 3.3.6, Figure 28A). This observation was in agreement with the impaired ability of the mutant to associate with the membrane via PI(4)P-PHD interaction (Levine and Munro, 1998, 2002; Ngo and Ridgway, 2009; Goto et al., 2016).

The protein began to desorb slowly from bilayers with PI(4)P soon after reaching a threshold bound mass during the course of the injection. This dissociation of membrane-protein complex hints strongly towards a possibility of protein-mediated extraction of PI(4)P molecules from the bilayer. The bilayer is gradually depleted of PI(4)P and the protein molecules arriving at the sensor during the latter part of the injection sensed a membrane devoid of its ligand. Consequently, wtOSBP showed little affinity for the PI(4)P-striped DOPC membranes. In agreement with this explanation, the time taken by the protein to reach the maximum adsorbed mass depended reciprocally on its injected concentration when the mol% of PI(4)P was kept constant in the bilayer (see Results, Section 3.3.4, Figure 26C). Beyond the protein concentration of 0.25 µM, the rates of association increased steadily in a dose-dependent fashion and were always higher than the dissociation rates. The gradual depletion of wild-type protein mass from
PI(4)P-containing bilayers, however, should not be compared with the rapid desorption of wtOSBP from neutral DOPC bilayers at the end of protein flow. This was arguably caused by bulk phase difference in the sample flowing over the chip when only a small amount of protein bound to the membrane.

Figure 34: Proposed events occurring during the interaction of wtOSBP with DOPC bilayers containing PI(4)P. The illustration depicts various equilibria that exist at the protein-membrane interface during the flow of wtOSBP over immobilized PI(4)P-containing bilayers. The association and dissociation of protein observed on the DPI is a culmination of three separate events defined by six independent rates.

My attempt to determine the rate constants $k_a$ and $k_d$ was challenged by the lack of any existing model that fits such protein behaviour. The closest resemblance was found with non-linear association then dissociation kinetics which assumes association of ligand with protein that reaches a plateau after a certain period of time, followed by dissociation that is initiated either by adding an antagonist or by massive dilution. This was not suited for the DPI curves because both adsorption and desorption were observed during the steady flow of protein over the membrane surface. This was further
complicated by the fact that the DPI only showed a global response from the events occurring at the protein-membrane interface which could be described by three distinct equilibria: i) association ($k_a$) and dissociation ($k_d$) of virgin protein, ii) uptake ($k_{on}$) and delivery ($k_{off}$) of PI(4)P, and iii) dissociation ($k_d$) and association ($k_a$) of PI(4)P-bound protein (Figure 34). Therefore, the true $k_a$ and $k_d$ could not be determined.

More mass per mm$^2$ of wtOSBP bound to bilayer surfaces with PI(4)P, unlike sterol-containing membranes. The time taken by wtOSBP to saturate the membrane increased in direct proportion to the mol% of PI(4)P. At a particularly high density of PI(4)P, for example 8 mol%, there was a complete absence of protein dissociation from the bilayer during the course of the injection. It may be that, past a critical concentration of PI(4)P, the dimeric OSBP (Dawson et al., 1989a; Lehto and Olkkonen, 2003) can bind to two molecules of PI(4)P simultaneously, which can possibly hinder lipid extraction and protein desorption from the bilayer surface.

4.5. Evidence of PI(4)P-extraction from membrane by OSBP

OSBP-HH/AA, unlike its wild-type counterpart, showed significantly higher adsorption to PI(4)P-containing bilayers compared to neutral DOPC bilayers. The mutant, devoid of any PIP-extracting ability, couldn’t leave the bilayer but remained strongly anchored via its PHD, long after the injection ended (see Results, Section 3.3.6, Figure 28B). Using the theoretical values of maximum possible mass of protein that could occupy the membrane surface, shown in Table 2, the actual surface coverage by OSBP was calculated based on the peak adsorbed masses obtained during the experiment. The behaviours of wtOSBP and OSBP-HH/AA were compared in
Table 3. The coverage, as expected, was lowest in the case of neutral DOPC bilayers due to the low affinity of the protein for bilayers lacking anionic phospholipids (de Saint-Jean et al., 2011).

Table 3: Coverage of DPI sensor surface by OSBP. Reported are the percent coverage of a lipid bilayer by OSBP based on maximum bound mass of protein/mm$^2$ of the chip surface, as observed experimentally. Reported masses are means ± S.E.M. of triplicate trials.

<table>
<thead>
<tr>
<th>Bilayer Composition</th>
<th>wtOSBP</th>
<th>OSBP-HH/AA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Mass (ng/mm$^2$)</td>
<td>% Coverage</td>
</tr>
<tr>
<td>DOPC only</td>
<td>0.5 ± 0.4</td>
<td>10.0</td>
</tr>
<tr>
<td>DOPC + 1 mol% PI(4)P</td>
<td>0.7 ± 0.2</td>
<td>34.0</td>
</tr>
<tr>
<td>DOPC + 2 mol% PI(4)P</td>
<td>1.0 ± 0.1</td>
<td>24.7</td>
</tr>
<tr>
<td>DOPC + 4 mol% PI(4)P</td>
<td>1.6 ± 0.3</td>
<td>30.7</td>
</tr>
<tr>
<td>DOPC + 6 mol% PI(4)P</td>
<td>3.4 ± 0.7</td>
<td>63.4</td>
</tr>
<tr>
<td>DOPC + 8 mol% PI(4)P</td>
<td>3.2</td>
<td>60.4</td>
</tr>
</tbody>
</table>

Since purified OSBP exists as homodimer (Dawson et al., 1989b), the unusually high membrane surface coverage by the double-histidine mutant at low mol% of PI(4)P could be explained as follows: a PI(4)P-poor membrane will allow only one OSBP molecule of the dimer to anchor with the ligand while the other molecule stays ligand-free due to scarcity of PI(4)P in its vicinity. In such a scenario, the binding stoichiometry becomes 2:1 (protein to ligand) which closely agrees with the observed 150% coverage at 1 mol% PI(4)P. As the membrane gets richer in PI(4)P, both members of the OSBP dimer attach themselves to adjacent PI(4)P molecules, now available in abundance in the bilayer, thereby shifting the stoichiometry to 1:1. This explains the observed 100% surface coverage at higher PI(4)P density. In contrast, wtOSBP could never achieve a near 100% coverage when it
interacted with the bilayers that contained physiologically relevant amounts of PI(4)P. This was caused by constant ligand extraction and departure of the ligand-bound protein from the bilayers. Taken together, the data from wtOSBP and OSBP-HH/AA demonstrated that OSBP was capable of recognizing the PI(4)P ligand in surface-adsorbed bilayers, associating with the membrane in a ligand concentration-dependent manner and extracting PI(4)P before departing from the membrane surface. This function was hampered when the ability of the protein to recognize PI(4)P was compromised.

4.6. Factors responsible for the failure of immunodetection of protein-extracted PI(4)P

My attempts to detect PI(4)P extracted by wtOSBP by immunodetection, remained unresolved for several reasons. The DPI effluent, containing the running buffer that was injected as a control over the adsorbed PI(4)P-containing bilayer, tested positive for PI(4)P when blotted on nitrocellulose membrane and probed with anti-PI(4)P antibody (see Results, Section 3.3.5, Figure 27). This was a technical artifact because it was highly unlikely for PI(4)P, which was incorporated into the adsorbed bilayer, to leach out into the buffer stream. Moreover, the intensity of the blot signals, for control and actual experiments conducted with 4 mol% PI(4)P in the bilayers, were unexpectedly higher than 10 nmol PI(4)P standard. This couldn’t have happened because the amounts of PI(4)P extractable from the adsorbed membranes were in the range of 1 – 6 pmol. Therefore, it was concluded that the signals noticed on the fat-blots were caused by the residual lipids, including PI(4)P, sticking to the inner walls of fluidics tubing, that got included in the collected effluent despite the best efforts to eliminate them by rigorous washes.
A second attempt made to confirm PI(4)P extraction, by means of detecting loss-of-PI(4)P signal in bilayers exposed to wtOSBP when compared to a buffer control, also remained inconclusive for two reasons. First, the amounts of PI(4)P present on the membranes were too low for this antibody-based assay to detect. Second, the ordinary methanol-chloroform-based PI(4)P extraction protocol, followed for this assay, might have resulted in the loss of PIP by spontaneous hydrolysis. These methods, originally proposed by Bligh & Dyer et al. (Bligh and Dyer, 1959) and Folch et al. (Folch et al., 1957), have been criticized for poor recoveries of phosphoinositides and other polar acidic lipids (Pettitt et al., 2006). PIP extraction under mildly acidic condition using citric acid/sodium phosphate buffer (pH = 3.6) and butan-1-ol can improve recovery by minimizing phosphoinositide deacylation and possible phosphate loss commonly encountered during acidification by HCl (Pettitt et al., 2006; Zhao and Xu, 2010).

4.7. Examining monomeric sterol-binding by OSBP using fluorescent sterol analog

Judging by the interaction of 25-hydroxycholesterol with the ORD (Figure 35), 22-NBD-cholesterol seems capable of inserting its steroid ring into the hydrophobic tunnel of the ORD. This, however, can give rise to some steric hindrance between the bulky NBD group and the N-terminal lid. But the fact that NBD’s fluorescence is greatly reduced in polar solvents, due to the formation of hydrogen bonds between the solvent and fluorophore (Lin and Struve, 1991), makes it a great tool for investigating lipid-protein interactions and transport of lipid molecules between organelles (in vivo) and artificial model vesicles (in vitro). 22-NDB-cholesterol was, therefore, chosen for the assays described in this thesis.
Figure 35: Crystal structure of 25-hydroxycholesterol-bound Osh4. A cartoon representation of the yeast ORP Osh4 bound with 25HC (blue with O-atom coloured red) is shown. Sterol molecule binds within the hydrophobic tunnel formed by antiparallel \( \beta \)-sheets (yellow). Ligand uptake and exchange is controlled by a flexible N-terminal lid (dotted circle). The images are prepared in PyMOL (ver. 1.7.4.5) from the 1HZX structure solved by X-ray diffraction at 1.5 \( \text{Å} \) resolution (Im et al., 2005). A structural comparison of 25-hydroxycholesterol with 22-NBD-cholesterol, showing the relative position of 25-hydroxyl (red) and 22-NBD (blue) groups with respect to cholesterol, is also depicted here.
The binding of the fluorescent cholesterol with both wtOSBP and OSBP-HH/AA followed a distinct hyperbolic response with respect to the concentration of added ligand (see Results, Section 3.4.1, Figure 29). The $K_d$ of 22-NBD-cholesterol for wtOSBP was 37.3 ± 5.1 nM. This can be compared to the saturable binding of radiolabeled [$^3$H]25-hydroxycholesterol with OSBP at a $K_d$ of 28 ± 5 nM, reported by Ridgway and co-workers (Goto et al., 2012) and [$^3$H]22(R)-hydroxycholesterol with ORP2 at $K_d$ of 14 nM, reported by Olkkonen and associates (Hynynen et al., 2009). A significantly lower affinity of Osh4 towards cholesterol, evident from a $K_d$ of 300 nM, was reported by Im and colleagues (Im et al., 2005). Since Osh4 is structurally much simpler than OSBP, it is not surprising to believe that the additional domains in the latter have evolved to stabilize the overall structure of the cholesterol-ORD complex, enabling the protein to participate in the trafficking of a wider range of sterols. The affinity of 22-NBD-cholesterol for OSBPHH/AA ($K_d = 34.0 ± 2.6$ nM) was similar to that for wtOSBP. It was in agreement with the fact that this mutant had intact sterol-binding ability and therefore, its affinity for cholesterol should not be affected due to the existing His → Ala mutations.

An interesting outcome of the monomeric sterol binding assay was the binding of 22-NBD-cholesterol with bovine serum albumin (BSA) with an affinity similar to that for OSBP. Albumin is one of the most abundant proteins present in the blood serum. It is known to be involved in cholesterol metabolism by acting as a shuttle to enhance cholesterol efflux from cells and incorporation of cholesterol into extracellular acceptors, such as lipoproteins (Fielding and Moser, 1982; Zhao and Marcel, 1996; Sankaranarayanan et al., 2013). In spite of such evidence, albumin is not known to directly associate with cholesterol (Haberland and Reynolds, 1973). It can, however, bind a multitude of steroid and other lipophilic hormones with low affinity ($K_d$ values in mM – µM range) (Baker, 2002). With that in mind, it was normal to
expect 22-NBD-cholesterol to bind non-specifically with BSA. The non-specific nature of such an association was verified by the competition assays wherein the natural sterols were unable to displace 22-NBD-cholesterol from the binding sites of albumin but quite effectively competed with the fluorescent sterol analog for the conserved ORDs of both wtOSBP and OSBP-HH/AA (see Results, Section 3.4.2, Figure 30). The $EC_{50}$ for competitive binding was three times higher for cholesterol than 25-hydroxycholesterol. This was in agreement with the higher affinity of oxysterol for OSBP than cholesterol documented by other groups (Dawson et al., 1989a; Ridgway et al., 1992; Wang et al., 2002, 2008).

4.8. Monitoring OSBP-mediated sterol transfer by FRET

Transfer of the fluorescent cholesterol analog to acceptor vesicles by OSBP was monitored by measuring FRET between 22-NBD-cholesterol and $N$-Rhod-PE. The NBD group exhibited a low initial fluorescence and the decay in its signal recorded at 535 nm, caused by energy transfer to Rhodamine, was not appreciable as evident from the traces that remained essentially “flat” (see Results, Section 3.4.3, Figure 32, B and D, insets). Due to the bulky nature of the NBD group, the fluorescent cholesterol analog, 22-NBD-cholesterol, may find itself in a rather solvent exposed environment within the ORD of OSBP than its natural counterpart (Figure 35). Therefore, when delivered to the acceptor membrane, the NBD fluorescence may increase substantially due to its transition to a more hydrophobic milieu. Although the emitted energy should be quenched by Rhodamine due to FRET, the fact that there wasn't a prominent decay could be explained by the prediction that the NBD traces were actually displaying a fluorescence gain overlapped with decay due to sub-optimal or inefficient quenching by Rhodamine, resulting in an overall “flat” response. In essence, the poor
reporting of the NBD fluorophore when trapped inside OSBP makes it a poor tracker of ligand transfer. Therefore, the kinetics of the transfer was defined by the fluorescence emission of Rhodamine at 581 nm upon excitation of NBD group at 469 nm.

The results showed similar transfer rates for OSBP-HH/AA when compared to wtOSBP for vesicles containing DOPC, 3 mol% N-Rhod-PE and 4 mol% PI(4)P. Also, the total amount of ligand transferred by the wild-type protein was significantly higher than that by the double histidine mutant. These outcomes can be explained by the fact that due to impaired PI(4)P recognition, OSBP-HH/AA could not exchange its ORDP-bound sterol for PI(4)P at the surface of the membrane. Therefore, it could spontaneously deliver, albeit rapidly, a smaller amount of sterol into the acceptor vesicle. wtOSBP, on the other hand, delivered a substantial amount of cholesterol, presumably by exchanging it for PI(4)P. However, the intensity of emitted fluorescence could not reach a plateau for either protein-mediated sterol transfer within the observed duration of the assay and therefore, requires a longer period of monitoring in future (see Results, Section 3.4.3, Figure 32, B and D).

In case of wtOSBP, lack of any significant difference in either the transfer rate or the amount of transferred ligand upon incorporation of PI(4)P in the acceptor membrane, was rather puzzling. At the estimated 1:1 molar ratio of protein to PI(4)P, under the experimental conditions, the wild-type protein was supposed to occupy more sites on the membranes compared to DOPC vesicles and exchange substantially more sterol for PI(4)P. One should be cautious while drawing meaningful conclusions from the transfer assay data because the signal obtained from FRET only represents energy transfer between two fluorophores, while the phenomenon occurring at the protein-membrane interface is a culmination of protein association, ligand exchange and protein dissociation. Each of these events can affect the proximity of the
two fluorophores required for FRET to occur and therefore, FRET may not be an absolute indicator of sterol transfer.

4.9. Concluding remarks

The work presented here demonstrates the utility of label-free surface microanalysis technique, such as DPI, in characterizing the behaviour of LTPs. The biosensor-based approach enabled direct investigation of the protein-bilayer interaction and displayed unique hallmarks of OSBP’s binding behaviour that were distinguishable from the trends observed in radiolabeled/fluorescent ligand binding studies widely reported in the literature. The goal of this project was to study the interaction of OSBP and its mutants with simple immobilized phospholipid bilayers, containing different molar amounts of sterols and phosphoinositides. In agreement with the currently proposed models of lipid-transport mechanism by OSBP (de Saint-Jean et al., 2011; Mesmin et al., 2013), the wild-type protein showed higher binding affinity for membranes containing anionic phospholipid, PI(4)P, as compared to neutral PC bilayers. Interestingly, the membrane-binding pattern of the protein remained unaltered when 25-hydroxycholesterol, a natural ligand of OSBP, was incorporated in the membrane. With the help of OSBP-RR/EE (characterized by a dysfunctional PHD and lack of phosphoinositide recognition) and OSBP-HH/AA (with compromised ability to extract PI(4)P from membranes), I provided a clear evidence of monomeric extraction of PI(4)P from adsorbed bilayers by wtOSBP.

The selectivity of OSBP in sequestering free sterols from solution was demonstrated by competitive binding of 25HC and cholesterol to the ORD saturated by 22-NBD-cholesterol. The ability of OSBP to deliver monomeric
cholesterol from solution to phospholipid vesicles was also demonstrated by the means of FRET between OSBP-bound 22-NBD-cholesterol and N-Rhod-PE in the acceptor vesicles. However, the kinetic measurements of this phenomenon require further validation to obtain a better appreciation of rates. Taken together, my observations provide a more comprehensive molecular level description of the function of this protein in intracellular sterol trafficking and lay a foundation for in vivo investigations.

In future, it will be of interest to utilize the DPI platform to examine if OSBP associates with membranes harbouring both sterols and the FFAT ligand VAP-A (an ER-resident protein), in a fashion similar to that of PI(4)P-containing membranes. It will also be worthwhile to investigate if wtOSBP extracts PI(4)P when sterol-loaded protein is injected over surface-immobilized membrane containing PI(4)P. If the sterol-loaded wtOSBP exhibits similar association profile as observed in case of free wtOSBP, then PI(4)P-extraction can be proven by performing a subsequent binding assay and injecting the protein on top of the PI(4)P-depleted membrane, without prior regeneration of the biosensor. OSBP’s ability to extract sterols in the presence of VAP-A should also be tested with OSBP mutant(s) with impaired oxysterol-binding ability. An example of one such mutant is OSBP-Δ432–435 that harbours a small deletion between the α-helical lid and β1-strand of the ORD (Bowden and Ridgway, 2008). The amino acids 409–459 map to the putative lid region of OSBP (Wang et al., 2008) and therefore, deletion of residues Glu-432, Leu-433, Ser-434 and Lys-435 causes impairment in sterol recognition and binding (Perry and Ridgway, 2006). Planar immobilized bilayers can be replaced with non-fused vesicles tethered to a chemically derivatized biosensor surface (Anderluh et al., 2005) for better comprehension of the role of membrane curvature on the binding affinity of OSBP. The membrane association of less characterized ORPs, such as ORP6, -7, -10 and -11, should also be studied using the techniques highlighted in this thesis.
BIBLIOGRAPHY


Anantharaman, V.; Aravind, L. The GOLD Domain, a Novel Protein Module Involved in Golgi Function and Secretion. *Genome Biol.* **2002**, *3*(5), research0023.1–0023.7.


Béaslas, O.; Vihervaara, T.; Li, J.; Laurila, P. P.; Yan, D.; Olkkonen, V. M. Silencing of OSBP-Related Protein 8 (ORP8) Modifies the Macrophage


Johansson, M.; Bocher, V.; Lehto, M.; Chinetti, G.; Kuismanen, E.; Ehnholm, C.; Staels, B.; Olkkonen, V. M. The Two Variants of Oxysterol Binding Protein-Related Protein-1 Display Different Tissue Expression


**Acta 1999, 1438 (1), 19–37.**


Singh, S. M.; Amulya, A.; Panda, K. Solubilization and Refolding of Bacterial


Üstün-Aytekin, Ö.; Gürgan, I. D.; Ohura, K.; Imai, T.; Öngen, G. Monitoring of the Effects of Transfection with Baculovirus on Sf9 Cell Line and


Xu, X.; London, E. The Effect of Sterol Structure on Membrane Lipid


APPENDIX
Supplemental Figure 1: Expression level of wtOSBP in S/21 cells at various stages of baculovirus infection. SDS-PAGE (A) and Western blot (B) analyses of expression of wtOSBP in baculovirus-infected S/21 culture, at the end of each day of incubation, are shown. Images represent the size-based separation of proteins obtained from the crude lysate of S/21 cells at 24 h (lanes 8), 48 h (lane 9) and 72 h (lane 10) post-infection with baculovirus at an MOI of 0.1. Molecular weight markers were loaded in lanes 1 and 7. Although full images are shown, the lanes of interest are highlighted in red. Therefore, readers are advised to ignore the results from lanes 2 – 6. Electrophoresis was performed on a 10% polyacrylamide gel and immunoblot samples were probed with mouse anti-OSBP mAb (dil. 1:200), as explained in Methods (Sections 2.5 and 2.6). Images were processed by Image Lab™ Software (ver. 5.2.1, Bio-Rad Laboratories Inc.).