Assay and array technologies for G-protein coupled receptors

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Surface immobilisation of GPCR molecular assembly

4.1 Introduction

4.1.1 GPCR array technologies

There is substantial interest in establishing solid supports capable of functional immobilisation of membrane proteins such as GPCRs. Applications primarily lie in protein microarray technologies for high throughput screening in the area of drug discovery. Due to inherent environmental requirements of membrane proteins, technologies establishing supported lipid bilayers (Knoll et al. 2000), immobilised lipid vesicles (Städler et al. 2006, Benkoski & Hook 2005), nanodiscs (Leitz et al. 2006) or high-density lipoprotein particles (Whorton et al. 2007), offer favourable interfaces to create arrays of membrane proteins. However, many of these techniques require the solubilisation and purification of the receptor prior to incorporation into prepared synthetic lipids. GPCRs are particularly difficult to isolate from their native environment, and for this reason, the strategy of using crude membrane extracts from cells overexpressing the GPCRs has been employed here.

The extensive number of known and potential GPCRs encoded in the human genome, and the even larger number of potential ligands, contributes to the desire to array these membrane proteins for high throughput screening (reviewed by Fang, Lahiri & Picard 2003), or sensing applications. There are a number of GPCRs that have been used as models for receptor immobilisation onto solid supports. These include the β2-adrenergic receptor (Neumann et al. 2002), the neurokinin-1 receptor (NK1R) (Martinez et al. 2003), the chemokine receptors CCR5 and CXCR4 (Navratilova, Dioszegi & Myszka 2006), the human delta opioid receptor (Alves et al. 2004), and the α2a-adrenergic receptor (Sen et al. 2005). But most commonly, bovine rhodopsin is used as it is the one of the only GPCRs which is naturally highly available and easily isolated (Karlsson & Stefan 2002, Bieri et al. 1999, Clark et al. 2001, Minic et al. 2005).

Solubilized receptors reconstituted into a lipid environment and native cell membrane
extracts containing the receptors (Hong et al. 2006, Fang, Frutos & Lahiri 2002b, Martínez et al. 2003, Neumann et al. 2002) have been used in attempts to attach these membrane proteins to a surface. Methods to control orientation of the membrane proteins involve the direct attachment of the C- or N-terminus of the receptor onto the surface via an affinity tag such as a biotin group (Bieri et al. 1999, Martínez et al. 2003, Neumann et al. 2002, Seguí et al. 2006), a histidine tag (Whorton et al. 2007, Sen et al. 2005) or GPCR antibodies (Neumann et al. 2002). Evidence shows that in solution, some GPCRs which are engineered with a tag for immobilisation retain G-protein coupling activity (Hayashi & Haga 1996, Whorton et al. 2007, Ott et al. 2005). With the exception of surface plasmon resonance (SPR) studies conducted using rhodopsin (Bieri et al. 1999, Komolov et al. 2006), there is little evidence to show associated, receptor-induced G-protein activity once the receptors are attached to a surface. It is the aim of this chapter to describe a method which could provide a platform to eventually introduce G-proteins into the array format. In this way it could be possible to monitor ligand induced conformational changes of the G-protein heterotrimer (for TR-FRET method to monitor G-protein interactions see chapter 3). Here, vesicles are used as the lipid support for the receptors. The rationale for this approach includes: the ease of preparation using crude membrane extracts (enabling minimal interference to the protein); the increased bilayer surface area and therefore maximization of the potential number of receptors within a given area on a planar support as compared to supported lipid bilayers; and the freedom of protein movement within the lipid bilayer when the vesicle is tethered rather than the protein itself.

4.1.2 Functional vesicle arrays

To introduce biological function into supported lipid systems there are two important factors to accommodate. Firstly, the native conformation and function of the protein must be retained and secondly the flexibility of the system must be adequate for physiological protein conformational changes or interactions to be retained. Studies involving synthetic vesicles (Christensen & Stamou 2007, Boukobza, Sonnenfeld & Haran 2001, Niemeyer et al. 1994, Stamou et al. 2003, Städler et al. 2004, Chaize et al. 2006, Pfeiffer & Hook 2004, Svedhem et al. 2003, Dusseiller et al. 2005, Yoshina-Ishii & Boxer 2003, Yoshina-Ishii et al. 2005, Niemeyer et al. 1999, Niemeyer 2004, Niemeyer 2007) have paved the way for research into functional vesicle array
platforms. Primarily, this technology is important in the immobilization of membrane proteins such as receptors and ion channels onto biosensing platforms. There are two main functional vesicle types that can be used for this application; the first is vesicles containing purified membrane proteins that have been reconstituted into lipids (either synthetic or isolated native lipids). The second is the isolation of native vesicles directly from the cell source. Lipids extracted directly from the host cell, commonly using chloroform/methanol mixtures, can serve as the lipid source for protein reconstitution (Neumann-Spallart, Pittner & Schalkhammer 1997). This strategy provides an environment for the isolated membrane protein which is as close as possible to that provided by the native cell. The lipid environment is thought to be critical for the proper functioning of the protein due to factors such as fluidity, which changes with the composition of membrane as further detailed in reviews by Mukherjee and Maxwell; and Simons and Vaz (Mukherjee & Maxwell 2004, Simons & Vaz 2004). Studies interested in establishing a membrane mimetic environment using lipids from the sources mentioned above, have utilized techniques such as sonication, freeze/thaw and extrusion technologies to create lipid vesicles. These vesicles are capable of incorporating a detergent solubilized membrane protein using techniques such as dialysis (Niu, Kim & Khorana 2002), detergent removal beads (Graneli, Benkoski & Hook 2007, Zagnoni et al. 2007), or other means of buffer exchange. Alternatively, solubilized protein preparations can be directly mixed with dried lipids (Contino et al. 1994). Some of the advantages that lie with the use of purified components include increased protein content per vesicle (potentially aiding in detection), and elimination of interfering components in the system such as other membrane associated proteins or carbohydrate molecules. Additionally, the stability of the pure proteins reconstituted into vesicles, would most likely exceed that of the native vesicles, which can be compromised if degrading enzymes are retained in the native preparation.

In this thesis, vesicles are used which are composed of the membrane extracts isolated directly from cells expressing the membrane protein of interest. This strategy was employed to minimize the risk of denaturing these sensitive proteins. This is because using crude extracts avoids solubilization and purification protocols for the membrane proteins (as reviewed in (Seddon, Curnow & Booth 2004)), which can prove problematic. Examples in the literature whereby membrane extract vesicles are prepared by separating the cell content, from the cell’s lipid component after cell lysis, include
techniques such as: hypotonic cell lysis and centrifugation (e.g. sucrose gradient) (Rao et al. 2002, Silin et al. 2006, Steck, Straus & Wallach 1970); Cytochalasin B (an agent used to destabilize the cytoskeleton-membrane interaction) induced membrane budding and vortexing (Pick et al. 2005), and cell disruption using high pressure followed by differential centrifugation. Further treatment of the cellular extracts can include extrusion (Bailey et al. 2009) and sonication (Minic et al. 2005, Seckler & Wright 1984) to produce more homogeneous mixtures of these membrane protein vesicles. Membrane protein budding experiments resulted in more control on protein orientation (Pick et al. 2005) than methods which relied on high pressure cell disruption and subsequent formation of membrane vesicles.

Vesicles have been used as a means of delivering surface immobilized bilayers (generally through vesicle rupture onto a surface or vesicle fusion into tethered bilayers), consisting of the extracts and associated membrane proteins (Rao et al. 2002, Minic et al. 2005, Zagnoni et al. 2007). Additionally, recent studies have shown the production of intact vesicular arrays (Silin et al. 2006).

Studies arising from Silin et al. and Rao et al. have reported the immobilization of the chemokine receptor CCR5 (a GPCR) carried in vesicles (approximately 300 ± 100nm in diameter) comprising lipid membranes isolated from COS-1 cells (Rao et al. 2002, Minic et al. 2005, Zagnoni et al. 2007). Additionally, recent studies have shown the production of intact vesicular arrays (Silin et al. 2006). Silin and coworkers used intact vesicles as the lipidic support of the CCR5 receptor utilizing the biotin/streptavidin and antibody/antigen interactions to effectively capture a functional CCR5 receptor. The successful vesicle immobilization via this antibody/antigen pair was monitored using surface plasmon resonance. Studies which also involve antibody directed immobilization strategies have been conducted using rod outer segment (ROS) membranes with associated bovine rhodopsin (Minic et al. 2005). These studies are important because they demonstrate the use of native membrane vesicles as hosts for GPCR surface immobilization, a strategy which was employed in this thesis.

The use of vesicles as supports for membrane proteins in biosensing applications provides an environment capable of allowing protein flexibility and movement within their native lipid environment. As such this could include the ability to monitor their
interactions with introduced associated proteins. The use of vesicles results in the compartmentalization of membrane fractions to isolated areas on a surface. Advantages in partitioning of membranes via a barrier (e.g. gaps in the hydrophobic environment of the lipid bilayer inhibits movement of the membrane proteins away from their site of immobilization), include allowing for the application of parallel experiments carried out on different receptors or receptor complexes (e.g. receptors with associated signalling proteins), within a single array or biosensor platform. This chapter describes the use of site specific immobilization of membrane vesicles containing GPCRs using complementary oligonucleotide sequences. This strategy has the advantage of site-directed immobilization that is applicable to heterogeneous array formats.

4.1.3 Oligonucleotide directed vesicle immobilisation

The concept of oligo-directed immobilisation of lipid vesicles is attractive because lipid vesicles provide the required hydrophobic environment for functional vitality of integral membrane proteins. Additionally, using complementary oligonucleotides gives rise to the ability to screen multiple vesicle populations, perhaps carrying various protein types, in parallel, on a single platform. The concept of a virtually unlimited amount of sequence combinations or “barcodes” (Battersby et al. 2002) and the ease in which stable oligonucleotide arrays can be manufactured, mean the rate limiting factor in this technology is producing stable lipid particles encapsulating or composed of functional proteins of interest. In contrast to oligonucleotides, proteins can be denatured when in contact with solid supports and, consequently there is an increased need to investigate appropriate surface chemistries which retain the functional integrity of the proteins. An advantage that lies with the use of vesicle arrays is that direct surface contact of the proteins themselves would not be necessary. Additionally, in the case of membrane proteins, the increased distance between the tethered bilayer and the surface would decrease the chance of any loss of membrane protein mobility and function often associated with proteins embedded in supported lipid membranes.

Oligo-directed immobilization of lipid vesicles was first introduced by Niemeyer and co-workers (Niemeyer et al. 1994, Niemeyer et al. 1999, Niemeyer 2004, Niemeyer 2007) and has since become a popular concept in the area of protein array technologies. A number of research groups have investigated the capture of lipid vesicles via this
Vesicles composed of pure lipid preparations tagged with an oligonucleotide have been captured on surfaces constructed using Molecular Assembly Patterning by Lift-off (MAPL) (Städler et al. 2004), or by spotting functional complexes (Städler et al. 2006, Chaize et al. 2006). The use of these vesicles as carriers of proteins has also begun to be investigated (Svedhem et al. 2003, Graneli, Benkoski & Hook 2007). In this chapter, Sf9 cell membrane extracts containing the H1-histamine or M2-muscarinic receptors have been prepared by differential centrifugation and attached to specific regions on a surface using complementary oligo sequences (for details on receptor extracts, see chapter 2).

### 4.1.4 Alternative protein scaffolds

While vesicles provide attractive environmental and spatial features as hosts for immobilizing membrane proteins, there have been reports of instability problems of vesicles in various situations, such as the presence of increased water content or various buffer compositions (Shoemaker & Vanderlick 2002). However, alternative lipid-like environments composed of different surfactant and lipid mesophase forms, such as the cubic and hexagonal phases, can also be produced. These offer more stability, a larger surface area, and aqueous channels throughout the structure which provide accessibility to both sides of the continuous lipid bilayer. These discrete lipid particles are formed.
from a bulk mesophase of an appropriate lipid or amphiphilic molecules and are termed cubosomes and hexosomes (Larsson 2000).

### 4.1.4.1 Cubosomes

Lipids and amphiphilic molecules possess the inherent capacity to adopt a variety of so-called mesophases in response to variations in temperature, pressure, molecular structure and concentration and composition of aqueous dispersing medium. The phase sequence, which is most commonly reported as a function of increasing amphiphile concentration, consists of; micelles, micellar cubic, hexagonal, bicontinuous cubic, lamellar, reverse bicontinuous cubic, reversed hexagonal, reversed micellar cubic, and reversed micelles (Kaasgaard & Drummond 2006). The lamellar phase which is the basis of the planar lipid bilayer of the vesicle structure is one such state. This phase, is most commonly adopted as the platform to accommodate hydrophobic proteins external to their cell environment. The bicontinuous phase consisting of a single continuous curved lipid bilayer forms a complex network with 3D cubic symmetry and continuous water channels. Various bicontinuous cubic phase forms, with the crystallographic space groups; Ia3d (gyroid surface), Pn3m (diamond surface), and Im3m (primitive surface), are shown in Figure 4.1.2 (Caffrey 2000).

![Figure 4.1.2 Cubic phase surface curvatures](image)

**Figure 4.1.2 Cubic phase surface curvatures.** Individual lipids are shown as small circles with adjoined curved lines, representing the polar headgroup and hydrophobic acyl chain respectively. The red and green regions represent water. Reproduced with permission from (Caffrey 2000).

Lipid phase behaviours (polymorphisms) are commonly mapped out via phase diagrams, the simplest of which varies water content (lyotropic) and temperature (thermotropic). Phase diagrams for lipids commonly found in the biological membrane (phospholipids, sphingolipids, glycolipids and sterols) have been investigated by a wide number of researchers and many can be found using a lipid index developed by Koynova and Caffrey (Koynova & Caffrey 2002). There are only a few polar lipids/surfactants known to have suitable phase behaviour for the formation of the cubic
phase. Furthermore, in order that such cubic phases can be utilized, they must exhibit properties such as stability in excess aqueous systems and existence at an acceptable temperature range (e.g. 4-40ºC). One such molecule which is commonly used to create bulk cubic phase is monoolein due to the conditions at which the phase transitions are achieved. In addition, more recent studies have investigated the phase behaviour of the phytantriol/water system (Barauskas & Landh 2003) (for examples of monoolein and phytantriol lipid phase diagrams, see appendix 6.3). In more complex systems, comprising more than one lipid/surfactant, or inclusion of additional buffer components or proteins, phase diagrams will shift, (e.g. ternary phase diagrams demonstrate changes with the addition of a third component within a lyotropic mixture). In addition, compositional and temperature requirements to reach the cubic phase may vary. For this reason, it is important to ensure that the correct phase is maintained within the range of the conditions and parameters of interest.

Bicontinuous cubic phase systems are becoming increasingly popular in studies involving drug delivery (Drummond & Fong 1999, Shah, Sadhale & Chilukuri 2001) and in crystallography applications (Caffrey 2000, Misquitta et al. 2004). The lipid cubic phase can be stable for long periods of time (months) (Lindblom & Rilfors 1989) and functional proteins can be incorporated within the membrane bilayers (Heberle et al. 1998) or within the aqueous channels (Ericsson, Larsson & Fontell 1983). Both of these properties make such a phase system worth investigating as a host for membrane proteins in array and/or biosensor applications. Studies whereby enzymes (such as glucose oxidase and lactate oxidase) have been entrapped in a cubic lipid matrix of monoolein and water have demonstrated the adaptability of this structure to create protein supports capable of immobilization onto transducer substrates (Razumas et al. 1994). Additionally, cholesterol oxidase immobilized in cubic phase matrices has been shown to be successfully used as a sensing element for detection of cholesterol (Ropers et al. 2001). Furthermore, the receptor-dependent activation of G-proteins has previously been demonstrated within the lipid cubic phase (Navarro, Landau & Fahmy 2002). The water content of the phase, the temperature, the pressure and the lipid composition, are all of importance to the stability of the cubic phase when increasing the protein loading (Nazaruk et al. 2008).

As mentioned previously, the strategy of compartmentalising the membrane protein
containing lipid support by using individual lipid particles such as vesicles, has favourable implications when monitoring separate reactions or biological events on the same platform. In the same way as vesicles can be used as reaction vessels (Christensen & Stamou 2007), or protein carriers, lipid particles organised in the cubic structure could provide a potentially more stable alternative with perhaps an increased loading capacity. The bulk cubic phase can be prepared as stable nanoparticle dispersions, termed cubosomes (Spicer 2005). The classic approach to preparing cubosomes involves mixing the amphiphile with water to first prepare bulk cubic phase, which is a viscous mixture. It has been previously reported that solubilised proteins can be mixed with the bulk cubic phase and become incorporated into the continuous bilayer (Caffrey 2000), although continuation into cubosomes has not as yet been reported to my knowledge. For production of the cubosomes, the bulk cubic phase gel is disrupted into nanoparticle dispersions most commonly by high-pressure homogenization or ultrasonication in the presence of a stabilising polymer (required to stop particle aggregation). Due to the high likelihood that these procedures could prove too harsh for proteins, alternative approaches attempting to minimise energy input required for disruption of the bulk cubic phase, are beginning to be investigated. These studies include the use of hydrotropic agents such as ethanol (Spicer et al. 2001, Zheng et al. 2003) to dissolve the viscous bulk mesophase. Then by addition of excess water, solubility is reduced and discrete particles are formed.

4.2 Materials and methods

4.2.1 Materials

Fluorescent ligands BODIPY-pirenzipine (excitation/emission) 558/568 nm (muscarinic receptor antagonist) and BODIPY-histamine (488/504 nm) (histamine receptor agonist) were purchased from Invitrogen. Talenzapine amine congener (TAC) was provided by the NIMH chemical synthesis and drug supply program and labelling with Alexa 546 NHS (Invitrogen, Australia) was carried out at room temperature for 2 hr at a molar ratio of dye:TAC of 1:1. Octadecyl Rhodamine was purchased from Sigma-Aldrich.
The graft copolymers poly(L-lysine)-grafted-poly(ethylene glycol) (PLL-g-PEG) and its biotinylated version PLL-g-PEG/PEG-biotin were purchased from SuSoS AG Switzerland. PLL-g-PEG consists in a Poly-L-lysine backbone [20 kDa] grafted with PEG side chains [2 kDa] in a grafting ratio of 3.5. The biotinylated PLL-g-PEG/PEG-
Biotin had a similar architecture with 50% of the side chains consisting of biotinylated polyethylene glycol [3.4 kDa]. For experiments conducted on QCM-D and Confocal Microscopy, oligonucleotide sequences were purchased from Eurogentec, Belgium, and were as follows: - BA1: 5'- CCC CCA TGG AAT CGT AA -3'; BB1: 5'- CCC CCT TCA GAG CAT AT -3' both with a 5' biotin modification; CA1`: 5'- CCC CCT AGT TGT GTA CAT TAC GAT TCC AT-3'; CB1`: 5'- CCC CCT-AGT TGT GAC GTA CAA TAT GCT CTG AA -3' both with a 5' tri-ethylene-glycol (TEG) cholesterol modification. For experiments using double cholesterol tagged oligonucleotides, the double cholesterol (indicated by a (d) infront of the cholesterol oligo name) is provided by 5'- TGT ACG TCA CAA CTA CCC CC-3' with a 3' tri-ethylene-glycol (TEG) cholesterol modification. For experiments conducted using the non-contact peizoarrayer and measured using the ArrayWoRx scanner, the same oligonucleotide sequences were purchased from Geneworks, Australia. Neutravidin (NA), Neutravidin oregon green (NAOG), Streptavidin conjugated to either Alexa fluor 633 (SA633) or Alexa fluor 532 (SA532) were purchased from Invitrogen. Cel-line® slides (Thermo Fischer Scientific) were purchased for experiments conducted using the non-contact peizoarrayer and measured using the ArrayWoRx scanner. In all cases ultrapure water (MilliQ Gradient, A 10 system, resistance of 18 MΩ/cm, total 4 ppb, Millipore Corporation, Switzerland) was used.

Rhodamine and biotinylated cubosomes were kindly provided by Dr. Tash Polyzos (CSIRO, Molecular and Health Technologies, Clayton, Vic, Australia). Phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecantriol) and Pluronic F127 were purchased from Sigma, Aldrich. Octadecyl Rhodamine B hydrochloride was purchased from Invitrogen and the biotinylated PEG/lipid conjugate (DSPE-PEG(2000)Biotin) was purchased from Avanti Polar Lipids (AL, USA).

4.2.2 Methods

4.2.2.1 Vesicle preparation

9/9 membrane preparations were diluted to a total protein concentration of 0.75 mg/ml. Following this, some preparations were then extruded using a hand-held mini-extruder (Avanti Polar Lipids, AL, USA) (for extruder image see Appendix 6.2) through a 1 μm polycarbonate membrane (Whatman, track-etch) followed by a 400 nm polycarbonate
membrane (Whatman, track-etch) at room temperature. In some cases this was followed again by a 200 nm polycarbonate membrane. GPCR/lipid vesicle preparations were tagged with an oligo sequence modified with a cholesterol moiety (CA1` and CB1`) at a concentration of 0.35 μM by incubation at room temperature for 10 min.

4.2.2.2 Cubosome preparation

Cubosomes prepared using a homogenizer were composed of 3.3% (w/v) phytantriol and 0.2% (w/v) pluronic F-127 (PEO-PPO block copolymer stabilizer) with 0.02% (w/v) of either octadecyl rhodamine or DSPE-PEG(2000)Biotin. Both the rhodamine labeled and biotinylated cubosomes were prepared and characterized by Dr. Tash Polyzos and Mr Scott Fraser (CSIRO, Molecular and Health Technologies, Clayton, Victoria). In an attempt to create cubosomes in the presence of proteins (in this case Neutravidin labeled with an Oregon Green fluorophore (OG)), cubosomes were prepared by sonication in the presence of ethanol. These cubosomes were composed of 3.3% (w/v) phytantriol and 0.5% (w/v) pluronic F-127 in 1, 2, 4 or 6% ethanol (v/v). The mesophase was prepared by mixing phytantriol and pluronic F-127 with ethanol in the presence of a soluble protein fraction (neutravidin OG) by a process involving ultrasonication with a probe sonicator (power level 3-4, Misonix Ultrasonic Processor, microtip ~1 mm diameter) for 1 x 5s, which resulted in a gel-like viscosity of the preparation. The cubic phase particle dispersion resulted after 3 x 5 second sonication pulses with 5 second intervals (with cooling) in the presence of 0.7% (w/v) pluronic F-127 stabilizer. The sample was placed on ice (cooling step) after each sonication step to prevent excess heat build up and maintain protein vitality. Particles were examined using cryo-TEM (see section 4.2.2.5) and Zetasizer nano ZS (Malvern, U.K.).

Rhodamine and biotinylated cubosome preparations (at 1/50x dilution) were tagged with an oligo sequence modified with a cholesterol moiety (CA1` and CB1`) or double cholesterol moiety (dCA1` or dCB1`) at a concentration of 0.35 μM by incubation at room temperature for 10 minutes. Incorporation of cholesterol oligos was monitored using site-specific capture of the fluorescent lipid particles.

4.2.2.3 Characterizing cell membrane extracts

Functional assays using radiolabels and radionucleotides to monitor receptor function
have been described previously (section 2.3 and 2.2.2.8).

4.2.2.4 Lipid extraction

To determine total lipid content in *Sf9* cell preparations (post urea treatment, as described in section 2.2.2.2), lipids were extracted using a modification of published protocols (Folch, Lees & Sloane Stanley, 1957). Membrane preparation (~ 700 μl) was extracted with 6:12:1 ratio of methanol; chloroform; MQ water. Mixtures were placed in an orbital mixer for 20 minutes then spun down at 650 x g for 10 minutes. The lower organic layer containing the extracted lipids was transferred into a clean, pre-weighed Kimble tube and dried under a stream of nitrogen at 40°C. Weight of extracted lipids was recorded in mg/ml of original extract.

4.2.2.5 Electron microscopy

Cryo-transmission electron microscopy samples were prepared by applying the vesicle or cubosome suspensions to lacey carbon grids. The lacey carbon provides a mesh support comprised of holes of various sizes, which allow for viewing of the sample through the thin vitreous ice formation within the holes during transmission electron microscopy (TEM). After applying the suspension to the grid, excess solution was blotted for 10-30 seconds using filter paper. The grid was immediately plunged into liquid ethane to form a thin vitreous layer containing the vesicles or cubosomes. The plunging process is carried out in a humidity chamber (80-95%) in order to avoid evaporation of the sample prior to its vitrification. Images were taken using low dose settings with an electron dose of less than 10 electrons per square Angstrom to minimise damage to the sample by the electron beam, on a FEI Technai 12 TEM operating at 120 kV and a magnification of 60,000x or 110,000x.

4.2.2.6 Surface preparation for site directed immobilisation

A schematic of the surface modification process is shown in Figure 4.2.1. The surfaces were first coated with a monolayer of biotinylated PLL-g-PEG (0.1 mg/ml) by adsorption from solution via electrostatic interactions between the positively charged PLL backbone and the negatively charged metal oxide surface. The biotin surface density could be varied using mixtures of functionalized and non-functionalized
polymers as described previously (Huang et al. 2002). If not stated otherwise, a mixture of 25% (w/w) PLL-g-PEG/PEG-biotin and 75% (w/w) non-functionalized PLL-g-PEG was used (12.5% of the PEG side chains biotinylated). Subsequently, the high affinity interaction of biotin and neutravidin was utilised to tag the biotinylated PEG chains with a biotinylated oligo (BA1 or BA2), 17 bases in length. The neutravidin (0.33 μM) and biotinylated oligonucleotides (0.35 μM) were pre-incubated for 10 minutes in HEPES preparation buffer (Table 2.2.1-1) prior to exposure to the biotinylated surface. Like streptavidin, neutravidin can bind to 4 biotin groups. Neutravidin is used as an alternative to streptavidin as it is carbohydrate free and has a neutral isoelectric point, which provides low non-specific background (Minic et al. 2005).

**Figure 4.2.1 Schematic representation of the immobilisation protocol:** Clean surfaces were modified by adsorption of PLL-g-PEG with 12.5% or 50% of the PEG chains terminated by biotin molecules (for QCM or array spotting, respectively). Further modification included immobilisation of the pre-formed neutravidin/biotinylated oligonucleotide complex. Vesicles containing GPCRs which were tagged with complementary oligonucleotides modified with a cholesterol moiety, were captured onto the oligonucleotide surface.

### 4.2.2.7 QCM-D

The QCM-D instrument (Q-Sense E4, Sweden) was used to measure changes in frequency (Δf) and viscoelasticity (measured as the dissipation factor, D) achieved through the adsorbance of layers onto the surface of an oscillating crystal. The dissipation factor is a dimensionless quantity which is the reciprocal of the Q-factor. The Q-factor is calculated from the decay time constant for the oscillating crystal.
Typically the D value for a quartz crystal is in the range of $10^{-6}$ (Rodahl et al. 1995). Using QCM-D the process of adsorption could be measured \textit{in situ}. The quartz crystal was coated with a thin layer of Nb$_2$O$_5$. The Nb$_2$O$_5$ coated crystal was cleaned by immersion in 2% (w/v) sodium dodecyl sulfate for a minimum of 30 mins, then rinsed in Milli-Q water and dried under a stream of nitrogen. The crystals were then exposed to O$_3$ plasma for 30 minutes prior to being mounted in the liquid-exchange cell of the instrument. Surfaces were prepared using modifications of published protocols (Städler et al. 2004). Surface modifications were made in degassed HEPES preparation buffer (Table 2.2.1-1) and stabilized at a temperature of 21-22°C. Resonance frequencies were measured at several harmonic overtones (overtone numbers = 3, 5 and 7). Surface modifications were carried out as described in section 4.2.2.6.

Surface coverage of the PLL-\textit{g}-PEG-biotin polymer could be deduced according to the Sauerbrey equation; $\Delta m = -C \Delta f n / n$ (where $C = 17.7$ ng.cm$^{-2}$.Hz$^{-1}$; $n =$ the resonance overtone number). The viscoelastic properties of the neutravidin/oligo complex and the GPCR vesicles diminish the accuracy of the Saurebrey equation. QCM-D data is used here as a qualitative method to monitor the adsorption of these subsequent layers. A change in buffer to TMN (table 2.2.1-1) in which the GPCR extracts are commonly assayed, was introduced (unless stated otherwise) prior to injection of the vesicles into the QCM-D cell. Vesicles, pre-incubated with a cholesterol modified oligo (with a sequence complementary to that on the surface), were introduced to the QCM-D chamber, and $\Delta f$ and $\Delta D$ were monitored.

\textbf{4.2.2.8 Pin and ring spotter}

A pin and ring spotter (GMS 417 arrayer, Affymetrix, USA) was used for the contact printing of functionalised spots (~120-130 μm in diameter) onto a polymer (PLL-\textit{g}-PEG-biotin) coated clean glass surface. To create the arrays, the neutravidin (4.2 μM) + BA1 or BB1 (see section 2.1) (4.4 μM) complex was diluted in 50% zeptoMARK spotting buffer (Zeptosens, Switzerland), and spotted onto the biotinylated polymer surface. Streptavidin633 (Invitrogen, Switzerland) was spotted as reference spots on either side of the oligo spots in the array.
4.2.2.9 Non-contact printing

The non-contact piezoarray printer (Perkin Elmer Life Sciences) consists of 4 pins which function in response to minute changes in a piezocrystal which results in picolitre droplets pushed from the tips. Slides used for all experiments using this arrayer were Cel-line® slides. These slides were used due to their hydrophobic coating surrounding 21 “wells” in which the glass surface is exposed (NB these exposed glass surfaces are termed “wells” within this thesis). The hydrophobic coating of the slides acts as a barrier for any aqueous droplets placed onto the bare glass areas, in effect, allowing the area to be a well for the aqueous environment remaining on the surface. To create the arrays, two methods were used. Firstly, the neutravidin (4.2 μM) + BA1 or BA1 (see section 4.2.1) (4.4 μM) complex was diluted in 15% (v/v) glycerol and spotted onto the biotinylated polymer surface. Secondly, PLL-g-PEG-biotin (0.1 mg/ml) was spotted onto the glass substrate, followed by a backfill with PLL-g-PEG. If required these arrays were re-spotted to overlay PLL-g-PEG-biotin spots with the neutravidin (4.2 μM) + BA1, or BA1 (4.4 μM) complex (see appendix 6.4 for schematic). Neutravidin oregon green (488nm) was used as reference spots on these slides.

4.2.2.10 Confocal laser scanning microscopy (CLSM)

Fluorescent GPCR ligands were used to monitor GPCR vesicle immobilisation and ligand binding function of captured receptors. BODIPY-pirenzepine (muscarinic receptor) and BODIPY-histamine (histamine receptor) were used at various concentrations in the experiments. To further demonstrate GPCR function, fluorescently tagged G-proteins (Gαi1Alexa; Gβγ2Alexa or Gβ1γ2Alexa) were used. Fluorescent experiments were performed using a Confocal Laser Scanning Microscope (ZeissLSM 510, Germany) using a 40x (LD, NA=0.7) objective. The CLSM was equipped with an argon (30 mW, 488 nm) and DPSS (10 mW, 561 nm) lasers which were used in these experiments.

4.2.2.11 ArrayWoRx scanner

The ArrayWoRx biochip reader (Applied Precision) is a 4 channel microarray reader equipped with the following filter sets: 1) Alexa488, which has an excitation of 480 ± 15 nm and an emission of 530 ± 40 nm; 2) Cy3, which has an excitation of 546 ± 11 nm and emission of 570 ± 10 nm; 3) Texas Red, which has an excitation of 602 ± 13 nm
and an emission of 631 ± 23 nm; 4) Cy5, which has an excitation of 635 ± 20 nm and an emission of 685 ± 40 nm. Vesicle and cubosome immobilisation was monitored using octadecyl rhodamine, a fluorescent amphiphile, which incorporated within the lipid bilayer of the vesicles or cubosomes. Additionally, GPCR presence and function in vesicle arrays was monitored using a fluorescent GPCR ligand, TAC-Alexa546 (muscarinic receptor ligand).

### 4.2.2.12 Data analysis

Measurements of elements within cryo-TEM images were analysed using Analysis software (FEI Technai 12 TEM). Data were analysed using Prism™ (GraphPad Software Inc., San Diego CA, USA). Data shown are mean ± SEM where sample number (n) is greater than or equal to 3, however, if the experiment was conducted in duplicate, data represents mean ± range of duplicates. Where error bars are not visible, they are within the data point symbol (unless otherwise stated). Apparent K_d and B_max values were calculated using non-linear regression analysis for one-site binding obtained from radioligand binding experiments on receptors in solution (see section 2.2.2.6). Quantitative analysis for images captured using CLSM were carried out using imageJ 1.37v (National institutes of health, USA) software. Quantitative image analysis for ArrayWoRx images was done using Genepix Pro6 software (Molecular Devices).
4.3 Results

4.3.1 Membrane extracts

The membrane extracts of the Sf9 insect cells were prepared as previously described (section 2.2.2.2). This procedure enables the isolation of membrane fragments from other cellular components such as the cell nucleus, DNA, RNA and soluble proteins (all of which would be removed during centrifugation steps). The membrane fragment isolated at 100,000xg would, however, contain not only the lipid constituents of the plasma membrane, but those of the much more predominant endoplasmic reticulum (ER) and mitochondrial membranes. Depending on cell type, the ER membrane can constitute more than \( \frac{1}{2} \) the total membrane surface area present in the cell (Albers et al. 2002). The membrane preparation will include receptors which have inserted into the ER membrane and those that are present in the plasma membrane. This membrane
fraction would also consist of membrane fractions originating from the lysosomes, golgi apparatus and intracellular vesicles and any proteins associated with these membranes. This leads to the obvious inclusion of these components into any assay or array applications, and the potential of these somewhat heterogeneous mixtures to induce artefacts in the resulting experiments. However, despite the origins of the lipids (and associated proteins) which are included within these membrane fragments, they are all amphiphilic in nature and thereby aggregate into shapes dictated by the hydrophobic effect. The crude nature of the extracts does not detract from the experiments in which they are used, but rather, provides an opportunity to adapt these methods to any membrane protein using a quick and easy protocol devoid of interferences from detergents and protein losses from purification protocols. In addition, these membrane preparations, show specific GPCR activity in functional assays described in chapters 2 and 3. The membrane extracts were prepared from cell culture which had either been infected with baculovirus encoding for a GPCR of interest (e.g. the H1-histamine receptor (H1R) or M2-muscarinic receptor (M2R)), or, as a control, was not infected with baculovirus and therefore did not express any GPCRs (previously described in chapter 2).

4.3.1.1 Protein concentration

Total protein concentration within the membrane extracts was determined using the Bradford protein assay (Bradford 1976) as has been previously used in liposome based assay systems monitoring liposome aggregation as a function of annexin (membrane associated protein) activity (Lee & Pollard 1997). Total concentration of protein within membrane extracts ranged from 5-20 mg per litre of Sf9 cells (with a cell concentration of 2 x 10^6 cells/ml).

4.3.2 Electron microscopy

Cryo transmission electron microscopy images seen in Figure 4.3.1 display a sample of membrane extracts from Sf9 cells expressing the M2R. The images demonstrate that the extracts are composed of a variety of vesicle shapes and sizes, with predominantly circular structures with an inner aqueous compartment (deduced by the low contrast within the structure). The darker edge of these vesicular structures is roughly 4-5 nm in width and this is believed to represent a lipid bilayer, as similar measurements have
been previously recorded for a lipid bilayer using cryo-TEM (Tahara & Fujiyoshi 1994). There are large vesicle structures with varied shapes and sizes within the cell membrane extract (Figure 4.3.1 and Figure 4.3.2). These irregular shapes (Figure 4.3.1 A (i)) could represent vesicle deformation, which may arise due to the confinement of the vesicle within the vitrified film (of defined thickness). Both unilamellar vesicles (Figure 4.3.1 B (ii)) and entrapped vesicles within vesicles (Figure 4.3.1 D (v)) can be found within the mixtures, the latter of which may provide limited accessibility of probe molecules (e.g. ligands and nucleotides) to proteins embedded within interior bilayers.

There also appear to be vesicles which are perforated around the bilayer (Figure 4.3.1 C (iv)). However, these apparent holes could potentially represent parts of the bilayer of slightly different composition and contrast than the rest. It is difficult to make that distinction with these present images. The components which make up the darker areas

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**Figure 4.3.1 Transmission electron microscopy images of non-extruded M₂R membrane preparations.** Cryo-TEM images of crude Sf9 cell membrane preparations containing the M₂R at a total protein concentration of 0.01 mg/ml, in a thin layer of vitreous ice over a lacy carbon grid. 110,000 x magnification and 120 kV. Scale bars represent 200 nm in these images. A-D represent images taken over various grids and grid spaces. A (i) large irregular shaped vesicles B (ii) Unilamellar vesicles; C (iii) dark areas, potentially protein aggregates and/or micelles or micro-vesicles (iv) Perforated vesicles "leaky" D (v) vesicles within other vesicles.
of the microscopy images (Figure 4.3.1c (iii)) are not able to be resolved, but could potentially represent protein aggregates, micelles or micro-vesicles. Micro-vesicles have been reported to be imaged as dark dots by cryo-TEM, but later resolved (by small angle neutron scattering studies) to be vesicle structures with a mean radius of 16-20 nm (Oberdisse, Regev & Porte 1998). Lowering the concentration of the solutions during grid preparation may show better separation of components (as can be seen in Figure 4.3.2).

![Image: Transmission electron microscopy images of non-infected cell membrane vesicles](image)

Figure 4.3.2 Transmission electron microscopy images of non-infected cell membrane vesicles: cryo-TEM images of crude Sf9 cell membrane preparations (not extruded) in a thin layer of vitreous ice over a lacey carbon grid. 110,000 x magnification and 120 kV. Both scale bars represent 200nm in these images. (i) non-infected membrane vesicle.

In Figure 4.3.2, the vesicle tagged with (i) shows a darker interior than the surrounding medium. This could be attributed to this large vesicle actually protruding out of the vitrified sample; an interpretation of similar cryo-TEM images of vesicles that has been described previously (Almgren, Edwards & Karlsson 2000).

### 4.3.3 Lipid extraction

The ratio of total protein concentration (mass/volume) as measured by the Bradford protein assay, to total lipid concentration (mass/volume) in the membrane preparation containing the H1R was 1:1.16 (lipid [mg/ml]: protein [mg/ml]). In membrane extracts prepared from cells which were not expressing the receptor, the ratio of total protein to total lipid was 1:1.24 (lipid [mg/ml]: protein [mg/ml]). These ratios cannot be expressed as mole ratios as the protein composition, and therefore molecular weights are not known. The composition of the Sf9 cell membranes which have not been infected with baculovirus have been reported as 23.0 ± 1.6% phosphatidylinositol (PI); 36.1 ± 1 %
phosphatidylethanolamine (PE); 35.1 ± 1 % phosphatidylcholine; and 4.6 ± 0.2% cardiolipin (Marheineke et al. 1998). This lipid composition only differed slightly for cells which had been infected with a recombinant baculovirus which was reported as 23.0 ± 0.3% PI; 32.4 ± 2.5% PE; 42.7 ± 0.6% PC; and 3.7 ± 0.3% cardiolipin (Marheineke et al. 1998). This shows that the vesicles prepared from non-infected cells and those prepared from GPCR expressing cells should behave in a similar manner when exposed to cholesterol moieties used in oligo-tagging. Therefore the non-infected membrane vesicles can be used as negative controls when measuring GPCR ligand binding to immobilized native membrane vesicles.

### 4.3.4 Tagging vesicles: cholesterol-lipid molar ratios

The concentration of lipid molecules within a protein concentration of 0.75 mg/ml is approximately equivalent to 62.6 μM for 750 μg/ml (647 μg/ml lipid) of membrane extract from infected cells (1:1.16 lipid:protein ratio) and 56.9 μM for 750 μg/ml (605 μg/ml lipid) of membrane extract from non-infected cells (1:1.24 lipid:protein ratio) (for calculations, see appendix 6.5). This equates to a molar ratio of cholesterol oligonucleotide:lipid of 0.0060 and 0.0064 for the infected and non-infected membrane extracts, respectively. The reported cholesterol:phospholipid ratio in whole Sf9 cells is 0.05 ± 0.001 and 0.04 ± 0.003, respectively, for baculovirus infected and non-infected cells (Marheineke et al. 1998), therefore the oligo tagged cholesterol content of the extracts makes up approximately 10% of the total cholesterol in the lipid mixtures. Using low levels of added cholesterol, minimal changes in the properties of the membrane bilayers (which may in turn influence protein function) are expected.

Reports of a cholesterol oligo:lipid molar ratio of 0.5 used with pure lipid vesicle immobilisation has previously shown successful immobilisation onto neutravidin-oligo complexes on a substrate surface containing a 1:1 neutravidin:biotinylated oligonucleotide complex (as was used throughout this investigation) (Städler et al. 2004), but these results indicate much lower molar ratios can be used in this experimental set-up. Although not shown here, further studies demonstrated that the mass binding to the QCM sensor (as depicted by change in resonance frequency) could be influenced by varying the concentration of the cholesterol oligonucleotide added to the membrane extracts (Bailey et al. 2009).
4.3.5 Capture of oligonucleotide tagged native vesicles

The Nb2O5 surface of the quartz crystal was modified in situ in the QCM-D flow cell (as depicted in Figure 4.2.1). Frequency and dissipation shifts were monitored over each step (Figure 4.3.3 (a) and (b) respectively). The first step involved the adsorption of the PLL-g-PEG/PEG-biotin onto the Nb2O5 surface. Surface saturation of the adsorbed PLL-g-PEG/PEG-biotin polymer was achieved within 40 minutes and resulted in a normalized change in the third frequency overtone (Δf3/3) of -37 ± 1 Hz and a dissipation (ΔD3) of 2.2 ± 0.1 x 10^-6. The second step of the surface modification was the introduction of the neutravidin/oligonucleotide complexes. Neutravidin/oligo complex binding to the biotinylated surface reached equilibrium within 1 hour. The adsorption resulted in a Δf3/3 = -30.7 ± 2 Hz and ΔD = 3.0 ± 0.3 x 10^-6. Both values are the mean ± SEM, n = 10 and are in agreement with previous reports (Städler et al. 2004). Before injecting the membrane extract vesicles, the buffer was exchanged from HEPES to TMN, a buffer well suited for GPCR preparations. This resulted in a small frequency change (<2 Hz) which stabilised before introduction of the GPCR vesicles.

Injection of the membrane extract tagged with a non-complementary oligonucleotide sequence (Figure 4.3.3 (3)) resulted in little non-specific adsorption onto the functionalised crystal surface (<5 Hz). However, when the extracts were tagged with the complementary sequence, a significant decrease in resonance frequency (87.67 ± 7.84, n = 4) (Figure 4.3.3a) was accompanied by a large decrease in viscosity (52.64 ± 8.68 x 10^-6, n = 4) as shown by an increase in dissipation in Figure 4.3.3b). These Δf and ΔD values are representative of the formation of a flexible layer of vesicles as reported previously (Städler et al. 2004). The viscoelastic properties of these adsorbed layers diminish the accuracy of the Saubrey equation used to convert Δf to Δmass, but instead, QCM-D measurements here are used as a qualitative method to monitor adsorption.
Figure 4.3.3 DNA specific Sf9 cell membrane vesicle immobilisation: Changes in frequency (a) and dissipation (b) as measured on a QCM-D upon immobilisation of a heterogeneous mix of vesicle sizes composed of preparations of Sf9 cell membranes containing over-expressed M2-muscarinic receptors. 1) PLL-g-PEG (12.5% biotinylated) is adsorbed onto a Nb2O5 coated quartz crystal; 2) Biotin-DNA complexed to Neutravidin is immobilised; 3) Surfaces are exposed to GPCR vesicles tagged with non-complementary oligonucleotide sequence; 4) Surfaces are exposed to GPCR vesicles tagged with complementary oligonucleotide sequence. *Washing ** buffer change.

The increase in dissipation measured once the extracts have been introduced, indicate that the viscosity of the surface has decreased. This can be attributed to the viscoelastic layer containing a large amount of water within the aqueous interior of the lipid vesicles present within the extract (as shown in Figure 4.3.1). The resulting mass on the surface and change in dissipation was similar for membrane extracts prepared from Sf9 cells expressing either of the receptor constructs (H1R or M2R) or those that were not infected.
with a baculovirus encoding for a GPCR (Figure 4.3.4). Additionally, two complementary oligonucleotide sequence pairs (A1 and B1) were compared and produced similar levels of binding (data not shown). Further injections of oligonucleotide tagged extracts into the chamber did not change the magnitude of the shifts, indicating that the surfaces were saturated.

![Figure 4.3.4 QCM data showing similar final immobilisation masses for all non-extruded receptors](image)

**Figure 4.3.4 QCM data showing similar final immobilisation masses for all non-extruded receptors**: Changes in frequency (overtone 3) measured by QCM-D upon capture of vesicles containing M₂-muscarinic receptor (blue line) or H₁-histamine receptor (red line) or non-infected membrane (black line). 1) Adsorption of PLL-g-PEG (12.5% biotinylated) onto a Nb₂O₅ coated quartz crystal; 2) Immobilisation of biotin-oligo complexed to Neutravidin; 3) Surfaces are exposed to GPCR vesicles which carry the complementary oligo sequence to that on the surface. 4) Non-specifically bound vesicles are washed from the surface with buffer. Spikes in curves represent buffer washes.

### 4.3.6 Extruding membrane extracts

The receptor membrane preparations contained particles which were larger than a micrometre in diameter (as seen using cryo-TEM) and a variety of shapes (including multilamellar structures). To obtain a more homogeneous mixture of unilamellar lipid vesicles, and potentially increase the stability of the vesicles used for array applications, the extracts were extruded sequentially through polycarbonate filter membranes of defined, decreasing pore sizes, at room temperature. The extrusion process, carried out by hand, required a considerable force to push the extract through the pores in the polycarbonate membrane filter. This indicated that the solution initially contained large particles and/or aggregates.
Figure 4.3.5 *Cryo TEM of extruded M\(_2\)R vesicles*: cryo-TEM images of M\(_2\)R vesicles, extruded through 400 nm polycarbonate membrane 67,000x magnification and 120 kV. Scale bar represents 200 nm.

The extrusion process not only substantially decreased the turbidity of the solution (as seen in appendix 6.2), it also decreased the content of total protein within the sample as measured by the Bradford protein assay (Bradford 1976) (see graph in appendix 6.6). It has previously been reported that the track-etch polycarbonate membranes can capture some proteins during the filtration process (although the report is not specifically referring to the extrusion process) (Kim *et al.* 1997), therefore the protein and potentially some attached lipid molecules may have indeed been lost during the extrusion procedure. The lipid classes present in the *Sf9* cell composition are composed of approximately 48% saturated to 52% unsaturated fatty acid chains (Marheineke *et al.* 1998). The fluid properties of the membrane rely on this lipid composition (including the presence of cholesterol, and in this case, quite low levels of cholesterol [cholesterol:phospholipid ratio = 0.05 ± 0.001 for infected *Sf9* cells (Marheineke *et al.* 1998)]). Fluid properties also rely on the temperature and water content at which the lipids reside. It has previously been reported that lipid solutions require a minimum pressure to pass through the pores in the membrane during extrusion, and this pressure is dependent on the lipid composition (Hunter & Frisken 1998). This minimum pressure may not have been consistently reached during the manual extrusion process. One, or a combination of these properties may contribute to the difficulty in the extrusion process at room temperature, as well as the apparent change in protein concentration of the cell extracts after extrusion.
4.3.6.1 Functionality of GPCRs in extruded extracts

Evidence that both the H\textsubscript{1}R and M\textsubscript{2}R remain functional once the extracts have been extruded through 400 nm polycarbonate filters was provided by saturation radioligand binding assays (as previously described in section 2.2.2.6) and the \textsuperscript{35}S-GTP\textsubscript{γ}S reconstitution assay (as previously described in section 2.2.2.8). Bmax values conveying number of receptor binding sites within the membrane preparation were calculated from a one-site binding curve fit for the extruded and non-extuded extracts and gave values of 12.6 and 10.9 fmoles/mg protein, respectively (Figure 4.3.6). Extruded extracts also demonstrated similar stimulation of G-proteins to non extruded samples as determined by [\textsuperscript{35}S]-GTP\textsubscript{γ}S binding (Figure 4.3.7). These results indicate that the total concentration of receptors included in these systems (as calculated from the Bradford protein assay) was very similar to that included in the experiments conducted on membranes prior to extrusion. Additionally, the results demonstrate that preparing smaller membrane vesicles via the extrusion process does not necessarily result in more receptors being exposed for activation (as was expected by producing unilamellar vesicles as opposed to multilamellar). It could also be assumed that the vesicles are quite “leaky” in both extruded and non-extruded samples. This allows the agonists, antagonists as well as reconstituted G-proteins, [\textsuperscript{35}S]-GTP\textsubscript{γ}S and other assay components to freely access the receptors on the inside and outside surfaces of the vesicles.
Figure 4.3.7 \[\text{\textsubscript{35}}\text{S}\text{-GTP}\gamma\gamma\gamma\gamma\text{S}\] functional reconstitution assay: Reconstitution of membrane preparations containing M\textsubscript{2}R or H\textsubscript{1}R. 20 nM G-protein subunits, G\textsubscript{ai1} and G\textsubscript{b4g2}, were combined with 0.05 mg/ml receptor preparation; 0.25 nM \[\text{\textsubscript{35}}\text{S}\text{-GTP}\gamma\gamma\gamma\gamma\text{S}\]; 10 \mu M AMP-PNP; 5 \mu M GDP. This reconstitution mix was then incubated with either buffer alone (basal, black column), an agonist (green column) or an agonist in the presence of excess antagonist (white column). a) H\textsubscript{1}-histamine receptor, stimulated with 1 mM histamine (agonist) and blocked with 100 \mu M pyrilamine (antagonist); n = 9. b) M\textsubscript{2}-muscarinic receptor, stimulated with 120 mM carbachol (agonist) and blocked with 100 \mu M atropine (antagonist); n = 9.

4.3.7 Site-directed immobilisation of extruded GPCR vesicles

Section 4.3.5 described the capture of non-extruded membrane extracts onto a surface (Figure 4.3.3), and that the extruded vesicles were also able to be captured oligo onto a surface. The difference between binding of extruded and non-extruded preparations is also demonstrated.

Oligo-tagged extruded membrane vesicles were also immobilised specifically onto oligonucleotide surfaces, as shown by the following features. 1) Large decreases in frequency (Figure 4.3.8a [dotted grey line (3)]); 2) increases in dissipation (Figure 4.3.8b [dotted grey line (3)]) once they are injected into the QCM-D chamber; and 3) very little change in frequency (Figure 4.3.8a [black line (3)]) and dissipation (Figure 4.3.8b[black line (3)]) when the same vesicles are introduced without the oligonucleotide tag. In a similar way to non-extruded extracts, the extruded vesicles originating from different cell sources produced similar levels of binding (data not
shown). However, in both the extruded and non-extruded samples, batch-to-batch variation was noted. The small batch-to-batch variations observed in the membrane extract binding properties measured by QCM-D (data not shown), could be the result of different numbers of oligonucleotides per vesicle for each batch which could lead to different binding efficiencies. Alternatively, varied binding properties could be the result of vesicle flattening could contribute, as previously observed with synthetic vesicles (Städler et al. 2004) or different cholesterol incorporation efficiencies due to slight variation of the lipid composition between infected and non-infected Sf9 cells (Marheineke et al. 1998).

Figure 4.3.8 Sf9 cell membrane vesicle immobilisation: Changes in frequency (a) and dissipation (b) as measured on a QCM-D upon immobilisation of <200 nm vesicles composed of preparations of Sf9 cell membranes containing over-expressed M2-muscarinic receptors. 1) Adsorption of PLL-PEG (12.5% biotinylated) onto a Nb2O5 coated quartz crystal; 2) Immobilisation of biotin-oligo complexed to neutravidin; 3) Surfaces are exposed to GPCR vesicles (extruded through 200 nm membranes) which lack any oligonucleotide sequence (black complete line) or to GPCR vesicles tagged with a complementary oligonucleotide sequence (grey spotted line). Unmarked peaks in data indicate a buffer wash.

Interestingly, the extruded membrane extracts (containing the smaller vesicles) showed
faster binding kinetics and higher levels of total coupled mass (as indicated by frequency change) as compared to the non-extruded membrane extracts containing the larger particle sizes (Figure 4.3.9).

![Graph showing changes in frequency for various sizes of vesicles]

Figure 4.3.9 QCM data showing changes in frequency for various sizes of vesicles: Changes in frequency (overtone 3) measured by QCM-D upon capture of vesicles containing M2-muscarinic receptor. As indicated on the graph, vesicles have either been extruded through 400 nm polycarbonate membranes or not extruded. T=0 represents time prior to injection of tagged vesicles onto the oligo modified surface. T~0.27 hours represents the point in time when vesicles, which were tagged with the complementary oligonucleotide to that on the surface, were introduced into the system. Mixtures containing the smallest vesicles appear to bind to the surface with the highest rate. Spikes indicate a buffer wash step.

The nature of the crude membrane extract used in the extrusion process makes it difficult to isolate the mechanism by which the extruded lipid particles have a faster, and increased, level of binding via the oligonucleotide tethers onto the complementary tags exposed on the surface. Although all of the components present in the membrane extracts prior to, and post-extrusion, were not investigated, some changes or loss of lipids, proteins or carbohydrates within the mixture during extrusion, could impact on the interaction of the vesicles with the surface. It could also be explained by the smaller particles diffusing faster and suffering less packing hindrance than the larger particles. Additionally, it has previously been reported that polysaccharide chains may contribute to decreases in diffusion rates onto a surface (Richter et al. 2007), and in this case, potentially alter oligonucleotide accessibility. Furthermore, the measure of a lower coupled mass when using the non-extruded vesicles, could indicate a flattening of the
vesicles onto the surface (Städler et al. 2004), although the viscosity (which has been shown to increase when vesicles are flattened) of these extracts remains unusually lower than extruded vesicles. A summary of the overall changes in frequency and dissipation for both extruded and non-extruded membrane extracts is presented in Table 4.3-1.

Table 4.3-1 A summary of the overall changes in frequency and dissipation for both extruded and non-extruded membrane extracts

<table>
<thead>
<tr>
<th>Addition onto oligonucleotide array</th>
<th>Extruded membrane preparations Vesicles &lt; 400 nm</th>
<th>Non-extruded membrane preparations Vesicles 50-1000 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FΔ3/3 (Hz)</td>
<td>ΔD</td>
</tr>
<tr>
<td>Complementary oligo-tagged vesicles</td>
<td>-289.9 ± 33.3</td>
<td>45.0 ± 7.7 x 10^-6</td>
</tr>
<tr>
<td>Non-complementary oligo-tagged vesicles</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Vesicles without oligonucleotides</td>
<td>-1.2 ± 1.7</td>
<td>1.5 ± 0.7</td>
</tr>
</tbody>
</table>

4.3.8 Spotted arrays

4.3.8.1 Site specific immobilisation

Spotted arrays composed of oligonucleotides were prepared using a pin & ring arrayer (GMS 417 arrayer, Affymetrix, USA) (Figure 4.3.10; Figure 4.3.11; Figure 4.3.12; and Figure 4.3.13), or a piezarrayer (Perkin Elmer Life Sciences) (results displayed in Figure 4.3.15 and Figure 4.3.16) on a glass slides displaying the biotinylated PLL-g-PEG polymer support. Oligonucleotide directed binding of the vesicles within both the non-extruded (Figure 4.3.10) and extruded extracts (Figure 4.3.11 & Figure 4.3.12) could be monitored using fluorescent ligands directed at the expressed GPCR. Both the BODIPY-pirenzepine ligand (Figure 4.3.11 and Figure 4.3.12) as well as a fluorescently tagged TAC ligand (appendix 6.7), have been used to successfully monitor vesicles containing the M_{2} muscarinic receptor. BODIPY-histamine, which was shown earlier to bind to the H_{1}-histamine receptor at high concentrations using the radioligand competition assays (Figure Figure 2.3.5), has also been shown here to bind to the vesicles containing the H_{1}Rs (Figure 4.3.10). H_{1}R containing vesicles (non-extruded) tagged with CA1’ were successfully immobilized (as shown by 1 μM BODIPY-histamine binding) onto BA1 (a biotinylated oligo complementary to CA1’) (Figure 4.3.10a), with minimal binding qualitatively observed on the non-complementary
surface (BB1) (Figure 4.3.10b).

Due to higher levels of binding observed on QCM-D measurements (Figure 4.3.9) using extruded membrane extracts as compared to those non-extruded, these were further investigated in the spotted array experiments (Figure 4.3.11 & Figure 4.3.12). Both membrane extracts used in Figure 4.3.11 & Figure 4.3.12 were extruded 11x through a 400 nm polycarbonate filter prior to incubation with an oligonucleotide tagged cholesterol moiety.

The necessity of the oligonucleotide presence on the surface is displayed in Figure 4.3.10 Confocal Laser Scanning Microscopy of H₂R containing vesicle attachment to complementary oligonucleotide modified surfaces. The H₂-histamine receptor was tagged with a) a complementary 32mer oligonucleotide or b) a non-complementary oligonucleotide sequence via a cholesterol moiety and were exposed to the surface. Vesicles composed of native Sf9 lipids from cells over-expressing the H₂-histamine receptor were visualised using 1 μM BODIPY-histamine (503/511 nm). (c & d) Schematic representation of immobilisation on spots containing c) complementary sequences to those displaying d) non-complementary sequence. Scale bars represent 100 μm.

Figure 4.3.11 Replicate dots show little non-specific binding on neutravidin: Spotted arrays were created with a GMS417 ring and pin arrayer. Green dots represent array spots displaying neutravidin/biotinylated oligonucleotide complex; yellow dots represent array spots displaying neutravidin only. Membrane extracts pre-incubated with cholesterol modified oligonucleotide (CA1`) were incubated on array slides for 3 hours post-sonication of tagged extracts for 30 seconds in a sonication bath. Extract immobilisation was visualised with the introduction of the fluorescent M₂-muscarinic receptor ligand, BODIPY-pirenzepine (2 μM). Fluorescence was measured at 575 nm after excitation at 540 nm. White scale bar represents approximately 100 μm.

The necessity of the oligonucleotide presence on the surface is displayed in Figure
4.3.11. This is shown by fluorescent ligand binding to spots displaying the biotinylated oligonucleotide, indicating capture of vesicles carrying M₂R, as compared with little fluorescence captured on neutravidin spots without the conjugated oligo. The necessity of the correct oligonucleotide sequence is demonstrated using the H₁R extracts in Figure 4.3.10. This image qualitatively displays a higher fluorescence coverage on complementary oligo spots (Figure 4.3.10a) as compared to spots displaying the non-complementary sequence (Figure 4.3.10b) (validating QCM-D results shown in Figure 4.3.3). Additionally, the necessity of the receptor presence is demonstrated in Figure 4.3.12 using non-infected membranes as the source of the vesicles immobilised on the surface in Figure 4.3.12d.

![Figure 4.3.12](image)

**Figure 4.3.12 Fluorescent ligand binding to vesicles containing the M₂-muscarinic receptor:** The presence of the M₂-muscarinic receptor is visualised using 100 μM BODIPY-Pirenzepine (558/568 nm)(Invitrogen). Simplistic schematic representations display vesicles a) over-expressing the M₂-muscarinic receptor, and b) not expressing the M₂-muscarinic receptor. The CLSM images show BODIPY pirenzepine binding to vesicles composed of c) Sf9 lipids from cells over-expressing the M₂R, and d) Sf9 lipids from non-GPCR-expressing cells. Vesicles concentrations were calculated using a [total protein] = 0.75 mg/ml and captured via a complementary DNA pair. Scale bars on all images represent 50 μm.

As previously mentioned, the immobilised mass, as determined by frequency changes in QCM-D was similar for the membrane extracts originating from different cell populations. This would indicate that these CLSM images are comparable and show that there is an approximate 35% lower fluorescence intensity when the receptor is absent in the vesicles (Figure 4.3.12b) compared to vesicles which contain the M₂R (Figure 4.3.12a).
Results showing receptor specific ligand binding were reproduced on arrays generated on waveguides for measurement on a zeptoREADER microarray scanner (as detailed in Bailey et al., 2009. Appendix 6.10). Results demonstrated (30%) non-specific ligand binding (on vesicles in which the receptor was not present) when using lower fluorescent ligand concentrations (1.25 μM and 2.5 μM) for the measurements.

Specific ligand binding was further confirmed using different batches of membrane extracts and different M2R fluorescent ligand (TAC-Alexa546) (appendix 6.7). Furthermore, the latter experiments were conducted using a different arrayer (peizoarrayer, Perkin Elmer) and microarray reader (ArrayWoRx, Applied Precision), further confirming the reproducibility of the array design.

These results demonstrate membrane extracts containing functional GPCRs tagged with oligonucleotides can be directed to spots on an array platform displaying the complementary oligonucleotide sequences. Array images, however, indicate some non-specific ligand adsorption to the vesicles (evident in the absence of the specific receptor) (as shown in Figure 4.3.12). This non-specific binding indicates that the BODIPY-pirenzepine conjugate could be lipophilic, a feature of fluorescent conjugates of GPCR ligands that has been described previously (Daly & McGrath 2003, McGrath & Daly 2003). Additionally, enrichment of the GPCRs within the extracts could aid in decreasing non-specific ligand binding by decreasing the amount of non-GPCR material within the extract. Such an improvement may result from adjusting the parameters associated with the preparation of vesicles, e.g. changing differential centrifugation steps.

Limitations associated with arrays which are reliant on fluorescent ligands for visualization, include the availability of the ligand for a wide range of receptors, and the difficulty in labelling small molecule ligands without compromising functional activity and binding affinity. An array format such as this, which allows extended freedom of the receptor for necessary interactions to occur, may provide the opportunity to avoid the use of fluorescent ligands for optical detection. It is envisioned that the incorporation of a generic fluorescent switch, capable of determining ligand activity, could potentially be incorporated into the vesicles. This switch has been discussed in
Chapter three and involves the interactions of the G-protein subunits. To determine whether the G-proteins can be coupled to the vesicles for future applications, such as a tethered TR-FRET signalling system, Alexa546 labelled Gαβγ heterotrimers were introduced into the tagged membrane extracts prior to introduction onto the oligonucleotide surfaces.

### 4.3.9 Specific G-protein immobilisation with GPCR vesicles

G-proteins labelled with Alexa 546 (Gαi1Alexa + Gβγ), associated with vesicles containing the M2R, were captured specifically on the complementary oligonucleotide surface (Figure 4.3.13a), with very little non-specific fluorescence on the spots displaying neutravidin alone (Figure 4.3.13b). Additionally QCM-D data (not shown here) displayed little non-specific binding on oligo modified surfaces when the vesicles were not tagged with the complementary sequence. From the above, it is likely that the fluorescent G-proteins (which do not bind to oligo spots alone, data not shown), are captured due to their association with the M2R vesicles tagged with the complementary oligo sequences.

![Image](image.png)

**Figure 4.3.13** Gαi1His Alexa capture with immobilised M2-muscarinic receptor in oligo-tagged vesicles: Gαi1His Alexa and β4γ2 were pre-incubated in the presence of 10 μM GDP with vesicles containing the M2-muscarinic receptor. Vesicles were tagged with CA1', cholesterol oligo and captured onto (a) spots functionalised with neutravidin + biotinylated complementary oligo (BA1). No spots were evident on (b) neutravidin alone. White scale bars represent 100 μm. c) Graphical representation of oligonucleotide specific capture of Gαi1Alexa⋅Gβγ associated with tagged M2R vesicles onto BA1 spots (solid red column) compared to neutravidin spots (chequered blue column). RFI – Relative fluorescence Intensity at 575 nm/Intensity of Strep633 Reference. *** P < 0.0001

The methodologies described here, whereby the GPCR and G-protein assembly are captured onto a surface via a lipid vesicle/extract, would potentially aid in establishing a generic immobilisation protocol for all GPCRs and likely also be applicable for other membrane bound proteins. It has been reported that the order of immobilisation of the receptor/G-protein complex onto Ni-NTA beads via a histidine tag, was important to the ability for the proteins to remain functional (Leifert *et al.* 2005b). Leifert *et al.* reported
that if the G-proteins (Gαi1 + Gβ1γ2His) were pre-bound to the Ni-NTA surface prior to the addition of the α2a-adrenergic receptor, the subsequent addition of activating ligand to the mixture did not result in activation of the G-proteins. However, when the receptor was pre-bound to the G-proteins and the complex was then immobilised onto the beads, ligand activation of the receptor could successfully be monitored through the binding of [35S]-GTPγS to the Gα subunit (Leifert et al. 2005b). Leifert et al. suggested this phenomenon could be due to conformational restrictions on the immobilised G-proteins. A way in which conformational restrictions to the proteins within the signalling complex can be avoided is if the proteins themselves are not bound to the surface, but the lipid environment within which they are carried in is bound to the surface initially. Preliminary results in Figure 4.3.13 indicate that the G-protein can be incorporated into the lipid vesicle and captured onto the surface using the complementary oligo immobilisation strategy. This is demonstrated by the use of Alexa546 labelled Gαi1 subunits pre-incubated with vesicles consisting of membranes containing the M2-muscarinic receptor. The mechanism by which the Gα subunit exists with the vesicles could be provided by a number of the following possibilities or a combination of these possibilities. The Gα Alexa 546 subunit (either with or without the Gβγ subunit), could be captured within vesicles during the brief sonication (30 seconds in sonication bath) performed in the presence of the cholesterol modified oligonucleotide. The Gα Alexa546 fatty acid moieties which occur at various sites within the protein (see section 1.3.1) could enable the protein to incorporate directly into the lipid bilayer of the vesicle or, alternatively, the fatty acid modification on the Gγ subunit could direct the Gαi1Alexa546 + Gβ4γ2 complex to the lipid bilayer, a mechanism that has previously been implicated as the method by which GPCR-G-protein interactions are initiated (Kisselev, Ermolaeva & Gautam 1994). The M2-muscarinic receptor, despite being in the un-ligated form may provide an attractive binding interface for the Gα Alexa 546 subunit (for more on Gα: receptor interaction interfaces, see section 1.2.1). Despite the mechanism by which the Gα Alexa546 subunit is captured (and whether it is alone or, more likely, complexed to the Gβγ subunit, although the location of the Gβγ subunit has not been monitored in this instance), it’s presence in the vesicles which are tagged with complementary oligonucleotides, was demonstrated by site specific capture on array spots displaying the complementary sequence (Figure 4.3.13). These images show an increased intensity at a central region of the array spots. This pattern has previously
been seen using the GMS 412 arrayer (data not shown), and could be attributed to a peak volume in deposited solution (carrying the neutravidin: biotinylated oligo complex) occurring when the solution remains momentarily in contact with the retracting pin during deposition. Despite this defect in spot quality, the resulting fluorescence is dependent on the presence of the complementary oligo, as the intermediate spots containing neutravidin alone (Figure 4.3.13b) do not display a similar level of fluorescence.

The vesicle based array technology demonstrated site-specific immobilisation and the capability of retaining active receptor amenable to functional array applications. There are a variety of methods by which the stability of liposomes/vesicles are being improved. These include manipulation of size, presence of inert surface groups, and inclusion of steric stabilisers such as PEG lipids (Lasic 1998). Here we demonstrated the ability to manipulate the size of the native vesicles using extrusion techniques (4.3.6).

Immobilisation of these vesicles composed of membrane extracts expressing a GPCR proved successful using the complementary oligonucleotide sequence capture technique. However, thorough investigation of the true functionality of these proteins (apart from ligand binding) was not demonstrated. This could be accomplished using the TR-FRET G-protein interaction assay described in chapter 3 of this thesis. However, these TR-FRET assays are most commonly carried out in solution, and it has not been shown enough proteins can be captured via this technique on a surface to enable any fluorescent changes to be monitored. GPCRs are notorious for their low expression levels and it would therefore be most beneficial to capture as many of these vesicles onto a surface as possible to perhaps amplify any response signal. One method by which this could be done has recently been described by Granèli and coworkers who demonstrated a multilayer vesicle structure composed of proteoliposomes containing a proton pump (transhydrogenase) (Graneli, Benkoski & Hook 2007).

In these vesicle experiments, a single cholesterol moiety was used to anchor the vesicle to the surface displaying the complimentary oligos. This single cholesterol interaction with the lipid bilayer would, however, most likely prove inadequate for a self sorting array since oligonucleotide exchange between vesicles has been reported previously.
Therefore, the method presented here enables only the creation of heterogeneous arrays by a sequential immobilization of the different vesicle populations. An answer to this problem can potentially be found in the work of Pfeiffer and Hook who have demonstrated that multiple cholesterol anchoring can provide a much stronger bilayer anchoring effect to the bilayer (Pfeiffer & Hook 2004). Although not shown in this thesis, preliminary data indicated that this double cholesterol/oligo tagging of the vesicles also resulted in the successful immobilisation of the GPCR containing vesicles onto a complementary surface. This double cholesterol method is further discussed in the next section (4.3.10.2). For mammalian cells, covalent coupling to azido groups could also provide an alternative (Douglas et al. 2007).

This immobilization strategy was shown to be well-suited for the immobilization of membrane extracts from non-infected cells as well as from cells over-expressing different GPCRs while preserving ligand binding specificity. This illustrates the flexibility and versatility of our approach. Furthermore, both extruded and non-extruded extracts could be immobilized with high-specificity.

As previously mentioned, the stability of vesicles is an issue which needs to be addressed (and is commonly monitored for applications such as drug delivery devices) (Lasic 1998). An alternative to vesicles, which may provide various advantages in the future of membrane protein array technologies, is investigated below.

### 4.3.10 An alternative membrane protein array platform

Instead of an array consisting of vesicles, this section discusses the use of lipid particles existing in a variation of the lamellar phase, known as the cubic phase. These particles have been termed cubosomes (Spicer et al. 2001). The preparation of the cubosome mixtures, provided by Dr. Tash Polyzos (CSIRO, Molecular and Health Technologies) were generally carried out using high energy shear forces provided by a high pressure homogenizer. An additional approach utilizing the formation of the cubosome particles made by sonication in the presence of ethanol is also described.

#### 4.3.10.1 Cubosome characterization

Cubic mesophases were prepared using 3.3% (w/v) phytantriol in the presence of
various concentrations of ethanol (1-6%) (v/v). Cubosomes were produced with short rounds of ultrasonication in the presence of 0.5% pluronic F-127. This study was done to determine the viability of this process to produce cubic phase particles capable of protein encapsulation. Unfortunately the presence of the fluorescently tagged, soluble protein (neutravidin oregon green) was not detected within the particle. This could have been due to either failure of the protein incorporating during the sonication, or the fluorescent proteins were not concentrated enough for detection. This has not been further investigated due to time constraints.

The number of small (<300 nm) particles that were visualised using cryo-TEM (Figure 4.3.14) increased as the concentration of ethanol used during preparation increased. This was further confirmed by peak distributions of particle sizes provided by light scattering measurements (Table 4.3-2). Here, a higher percentage of particles were sized as the smaller (~240 nm) particles, with increasing ethanol concentration (Table 4.3-2). Features of the internal structure of cubic phase previously described (Almgren, Edwards & Karlsson 2000, Lynch & Spicer 2005), can be seen in both bulk cubic phase (Figure 4.3.14b (i)) and cubosome particles (Figure 4.3.14b (ii)). The presence of the bulk cubic phase at lower preparative concentrations of ethanol (1% & 2%) indicated that perhaps phytantriol and pluronic stabiliser mixing was not adequate to enable stabilized particles after sonication.
Figure 4.3.14 Cubosome preparations prepared with a) 1% EtOH; b) 2% EtOH; c) 6% EtOH. Images display (i) cubic mesophase and (ii) cubosome particles. White scale bars represent 200 nm.

For use in functional array platforms, the method of preparation of the cubosomes must have minimal interference with protein activities. Ultrasonication is commonly used as a method of cell lysis, and in controlled circumstances could potentially aid in cubosome formation. The use of ethanol minimises the energy input required to produce the particles, but compositions of cubosomes that require low concentrations of the solvent would be best to ensure the proteins within remain functional.

Table 4.3-2 Cubosome size determination: light scattering. Zetasizer: nanoseries. n = number of measurements on particle solution.

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Peak intensity 1 diameter (nm)</th>
<th>Peak intensity 2 diameter (nm)</th>
<th>Ratio peak1:peak2 Reported from volume measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cubosomes (1% EtOH)</td>
<td>284.5 ±19.9 n = 3</td>
<td>5253 ± 128.3 n = 3</td>
<td>75.9:24.1</td>
</tr>
<tr>
<td>Cubosomes (2% EtOH)</td>
<td>226.2 ± 1.9 n = 5</td>
<td>5043 ± 147.5 n = 5</td>
<td>68.1:140.4</td>
</tr>
<tr>
<td>Cubosomes (4% EtOH)</td>
<td>227.4 ± 11.7 n = 5</td>
<td>5114 ± 294.8 n = 5</td>
<td>85:14.4</td>
</tr>
<tr>
<td>Cubosomes (6% EtOH)</td>
<td>239.5 ± 10.9 n = 4</td>
<td>4787 ± 555.4 n = 4</td>
<td>90.8: 8.4 (0.7% were of diameter 42.71nm as measured by a 3rd peak)</td>
</tr>
</tbody>
</table>
However, although sonication has previously been used for protein (enzyme) encapsulation, reduction in enzymatic activity associated with the sonication process has been reported (Tabata, Gutta & Langer 1993, Krishnamurthy, Lumpkin & Sridhar 2000). Further investigation into protein encapsulation techniques could follow the paths of either (1) integration of soluble proteins into bulk cubic phase prior to cubosome production by mixing, or (2) introduction of solubilised protein prior to cubosome production in the presence of a hydrotrope such as urea (Roy & Moulik 2003), which has already shown to be tolerated at certain concentrations by membrane proteins such as GPCRs (Lim & Neubig 2001).

While these results indicated that cubosome particles can be made using sonication, as has been described previously (Spicer et al. 2001, Zheng et al. 2003), the incorporation of proteins, which has been achieved in the past using cubic phase (Caffrey 2000, Misquitta et al. 2004, Razumas et al. 1994), has yet to be demonstrated within the cubosome particles. While protein capture was not achieved within the time limits of this thesis, the immobilization of these potential protein scaffolds using the complementary oligo system, was investigated.

### 4.3.10.2 Cubosome site directed immobilisation

Cubosome particles were used to further investigate the adaptability of the oligo immobilization strategy to produce self-sorting arrays of lipid/surfactant particles. Cubosomes used here (rhodamine and biotinylated) were prepared and provided by Dr. Tash Polyzos using rhodamine amphiphilic molecules (Octadecyl Rhodamine B hydrochloride, Invitrogen) to produce fluorescent cubosome populations (rhodamine), and biotinylated polymer conjugated to a lipid molecule (DSPE-PEG(2000)Biotin, Avanti polar lipids) was used to produced biotinylated cubosome populations. Cubosomes were composed of 3.3% (w/v) phytantriol; 0.23% (w/v) pluronic F-127 with 0.017% (w/v) of either octadecyl rhodamine or DSPE-PEG(2000)Biotin. Cubosomes were prepared using a high-pressure homogenizer maintained at 80°C.

Site directed immobilisation via complementary oligonucleotide sequences was accomplished using cubosome particles (Figure 4.3.15). Additionally, this could be monitored on more complex array patterns (see appendix 6.8). Tagging of the
cubosomes was accomplished at a molar ratio of 5.7 x 10^{3}:1 (phytantriol:cholesterol oligo). The cholesterol moiety was able to incorporate into the continuous bilayer structure of the cubosomes and effectively attach them to a site on the surface, specific to the sequence on the tag.

**Figure 4.3.15 Site specific immobilization of cubosomes**: The top row (5 blue spots) in each image represent the streptavidin647 reference spots in the array. The middle row (5 spots) display the biotinylated BA1 oligo; and the bottom row (5 spots) display the biotinylated BB1 oligo. a) Rhodamine cubosomes pre-incubated with CA1\(^{-}\) (cholesterol oligo) are captured on BA1 spots; b) Rhodamine cubosomes pre-incubated with CB1\(^{-}\) (cholesterol oligo) are captured on BB1 spots; c) Rhodamine cubosomes pre-incubated with dCB1\(^{-}\) (double cholesterol) are captured on BB1 spots. White scale bar represents 200 μm

Here, both single (Figure 4.3.15a & b) and double (Figure 4.3.15c) cholesterols were shown to accomplish specific oligo binding, showing significantly lower relative fluorescence intensity on spots displaying the non-complementary sequence (quantitative data is shown in appendix 6.9). The double cholesterol oligo was tested here for further use in the following heterogeneous array format. Increased tag stability within the bilayer is important when tag exchange could occur between two cubosome populations, as has previously been discussed with vesicle applications (Pfeiffer & Hook 2004). The double cholesterol is formed prior to incubation with the cubosome solution. This is accomplished by mixing equivolumes of CB1\(^{-}\) with the double (d) cholesterol shortened sequence. This enables the hybridization of the cholesterol-tagged 5\(^{\prime}\) end 15 nucleotides (after 5 x C spacer) of CB1\(^{-}\) and the cholesterol-tagged 3\(^{\prime}\) end 15 nucleotides (after 5 x C spacer) of the d-cholesterol tag. This provides a double cholesterol end to the remaining 12 nucleotides capable of hybridising to the complementary sequence on the surface.

### 4.3.10.3 Heterogeneous cubosome arrays

The attraction in using the oligonucleotide immobilization strategy is the potential to adapt it to a ‘self-sorting’ array platform. Here, the first step toward this application is investigated whereby two different fluorescently tagged cubosome populations are introduced sequentially onto an oligo array.
Due to the use of fluorescent streptavidin tagged cubosomes, background binding onto the PLL-g-PEG-biotin would be inevitable. The arrays, therefore, were produced with a PLL-g-PEG background. This was accomplished by using two rounds of spotting to produce the arrays (see multiplexed arrays in appendix 6.4). This involved the spotting of the PLL-g-PEG-biotin polymer onto the clean glass surface, followed by a backfill step of PLL-g-PEG, then a second spotting step using precisely the same co-ordinates as the first round to array the neutravidin/biotinylated oligo complex onto the pre-spotted biotinylated polymer. As seen in Figure 4.3.16, this resulted in an approximate 40-50% overlap of the spots and provided functionalised spots capable of oligo capture. The exposed PLL-g-PEG-biotin which remained from non-overlapped regions of the first spots, resulted in some background streptavidin647 binding. This was reduced with a blocking step of neutravidin alone. The cubosome populations used in this study were (1) the rhodamine cubosomes and (2) biotinylated cubosomes pre-incubated with streptavidin647.

Figure 4.3.16 Heterogeneous cubosome array. (A)(i) Array layout within wells on cel-line® slides consist of three rows of biotinylated oligos BA1 (green) and two rows of biotinylated oligos BB1 (red). (ii) Sequential addition of cubosome populations were sequentially incubated on the array, the first was the rhodamine cubosome population tagged with the double cholesterol oligo CA1`; and the second is a streptavidin647 labelled biotin cubosome tagged with dCB1`. (B) Array read using 595 nm laser on ArrayWoRx microarray scanner, showing rhodamine cubosome dCA1` capture; (C) Array read using 647 nm laser showing streptavidin Alexa647 labelled cubosome dCB1` capture.
Although research into this area is a work in progress, it is envisaged that the lipid/amphiphile composition of the cubosomes can be manipulated to produce the phase required at temperatures and water content suitable to particular applications. Additionally, specific lipid compositions which are required for physiological function of the incorporated proteins could possibly be accomplished.

The results presented here, demonstrated the proof of concept that these particles can be directed onto a surface via complementary oligonucleotides. This would be beneficial for systems requiring the use of two or more cubosome populations on the same platform. Cubosomes consolidate the favourable aspects of each of the lipid based systems that have been described throughout this thesis. Partitioning is provided by the discrete particles which are amenable to immobilization in a site-specific manner via complementary oligonucleotides. The particles are stable in an excess aqueous environment. The cubic phase provides an environment potentially capable of adsorbing and desorbing molecules and protein through aqueous channels that run throughout the structure. These channels provide access to both sides of the lipid membrane, potentially aiding access to binding interfaces present on both sides of a transmembrane protein such as a GPCR.

4.4 Chapter summary

This chapter demonstrates the site-specific immobilisation of native membrane vesicles carrying functional G-protein coupled receptors onto solid supports. Extrusion of the extracts provided a method to produce a homogeneous population of smaller vesicles, which, when appropriately tagged, showed greater coupling capacity to the oligo surfaces. Additionally, a new lipid structure, the cubosome, was investigated for its adaptability to this immobilisation strategy. Site-directed immobilisation was demonstrated for two fluorescent cubosome populations, tagged with double cholesterol oligos to minimise oligo exchange. This was achieved using sequential addition of the cubosome populations onto an oligo array displaying two oligonucleotide sequences.

Model lipid systems, such as vesicles and cubosomes, capable of stable and directed immobilisation onto a surface, can not only aid in the investigation of ligand-protein and protein-protein interactions as described throughout this thesis, but also could demonstrate important insights into protein-lipid interactions in a format amenable to
screen a large number of variations within the one platform.
5 Final discussion - applications and future directions

5.1 Summary of thesis

This thesis demonstrates the functional expression of the H₁-histamine, M₂-muscarinic and α₂a-adrenergic receptors of the G-protein coupled receptor family. Additionally, expression and purification of the Gα₁ (± hexahistidine tag), Gβ(1 or 4), and Gγ₂ (± hexahistidine tag) was also achieved. Expression of both the receptors and the G-protein subunits was carried out using the Sf9/baculovirus expression system.

In an attempt to create a homogeneous assay format capable of monitoring GPCR activation, and amenable to high-throughput screening, time-resolved fluorescence resonance energy transfer techniques (TR-FRET) were investigated. Specifically, the interaction of the G-protein subunits (indicative of GPCR signalling) was monitored using a terbium (long-lifetime) donor and an Alexa546 acceptor probe on the Gα subunit and Gβγ dimer, respectively. In a solution based format within microtiter plates, this assay demonstrated its ability to reveal the activation state of the G-protein heterotrimer, both in the absence (using AlF₄⁻) and in the presence (using agonist) of receptor.

The capability of the TR-FRET assay system to identify specific G-protein interactors, such as the tachyplesin peptide was demonstrated. This assay platform has the potential to screen therapeutics directed at the G-protein subunits, or proteins associated with G-protein signalling such as the RGS family of proteins.

In addition, the inclusion of the receptor into the TR-FRET assay system in the form of extruded vesicles (predominantly <400 nm diameter) enabled the detection of fluorescence changes resulting from the presence of agonist or antagonist ligands. The use of the membrane extract in the TR-FRET assay provided intrinsic difficulties presumably due to the particle size (and solution opacity), and interfering components within the extract. This commonly resulted in the loss of signal stability and
reproducibility. Problems were somewhat remedied by the extrusion of the membrane extract (thereby reducing particle size and perhaps slightly enriching the receptor concentration within the total protein content). However, while these results were not conclusive, due to the changes in net FRET being solely contributed by changes in donor emission, they do suggest the occurrence of the separation of the G-proteins (by a decrease in the net TR-FRET signal) upon addition of the specific agonist (carbochol) to the M2-muscarinic receptor within the vesicles. Additionally, the same fluorescence change was not observed when the agonist action was blocked by addition of an excess of a specific antagonist (atropine) to the M2-muscarinic receptor.

While these TR-FRET assays were conducted in solution, the homogeneous format of the assay could potentially be adaptable to an array based format. This thesis further described the capture of GPCR containing vesicles onto a solid substrate via the specific interaction between complementary oligonucleotides; (for experimental summary see Figure 4.2.2). The substrate composition has previously been described (Städler et al. 2006), and involves the use of a co-polymer PLL-g-PEG support to eliminate non-specific protein and/or lipid adsorption. This polymer support can contain PEG-biotin molecules which allow for further modification of the surface. The advantage of using the co-polymer was that the low-biofouling PEG brushes can be administered to a surface via direct adsorption from solution.

The vesicles were composed of the lipid component and associated proteins of Sf9 insect cell extracts. There are advantages and disadvantages which lie in the use of crude extracts. Advantages include the minimal handling of the membrane proteins of interest, as they do not require solubilization and isolation from their native lipid environment. However, disadvantages include the low levels of receptor within the vesicles compared with levels of other protein components within the mixture, and this may have contributed to detection limitations. Additionally, the use of fluorescent ligands to detect the presence of GPCRs on the surface proved problematic due to non-specific adsorption of the ligands. Nonetheless, the results clearly indicated that functional GPCRs were directed in a site-directed manner to pre-defined regions on an oligonucleotide array.

Alternative membrane protein array technologies in the form of cubosomes, were also
investigated for their adaptability to the site-directed strategy for immobilization. Using both the cholesterol oligonucleotides and the double cholesterol oligonucleotides to increase binding strength, the cubosomes were captured onto spotted microarrays displaying the complementary oligo sequence to that with which they were tagged.

5.2 Applications

Many disease processes involve aberrant or altered GPCR signalling and these receptors represent a significant target for medicinal pharmaceuticals (as discussed in section 1.4). Additionally, GPCRs have been implicated for their use in biomimetic sensors, e.g. members of the olfactory receptor family in the production of a sensor for smell (an electronic nose). A specific example of one such application is the rat olfactory receptor, Olfr226, which has been reported to respond to a compound mimic of TNT (Radhika et al. 2007), suggesting this receptor may have applications as the biorecognition element in explosive detection biosensors. Therefore, there is a need to develop appropriate GPCR biosensor and array platforms for the detection or screening of a variety of ligands.

Applications in compound screening at GPCRs include discovering new, more specific ligands for receptor subtypes, and thereby possibly minimising side-effects of drugs which undergo non-specific interactions with other receptors. Additionally, the screening of potential allosteric modulators (as discussed in section 1.2.1.1) to GPCRs may provide a new therapeutic strategy for GPCR targeting. Not only are the GPCRs themselves therapeutic targets, but the assay and array technologies described in this thesis may provide opportunities to monitor interactions between associated proteins such as the G-proteins and RGS proteins for their potential as drug targets.

5.3 Future directions

One of the main benefits of the oligonucleotide linkage concept is that a mixture of lipid particles (e.g. containing different GPCR subtypes), has the potential to self-sort onto an array whilst remaining in an aqueous environment (important to maintain membrane protein vitality). It could be of interest to use this immobilization approach to capture whole cells (previously demonstrated by Douglas et al. (Douglas et al. 2007) using Chinese hamster ovary cells and Jurkat cells), expressing different GPCR subtypes. This
could provide an alternative high-throughput screening tool for a number of applications such as; monitoring intracellular GPCR signalling responses (e.g. calcium levels or MAP kinase activation (Niedernberg et al. 2003)); GPCR complex conformation or location changes (e.g. fluorescence and bioluminescence resonance energy transfer measurements of GPCR or GPCR/G-protein or G-protein conformational changes (Lohse et al. 2008); and internalization of an activated receptor (as discussed in section 1.2.1) (Lee, Howell & Kunapuli 2006). The latter methods could have benefits in the elucidation of ligands for receptors for which the endogenous ligand is not known (orphan receptors), as knowledge of the ligand or the intracellular signal response is not required.

The use of the G-protein interaction as a generic signalling switch for receptor activation could be limited by specific GPCR/G-protein coupling. An improvement on this would be if it were possible to array a common set of G-protein heterotrimeric that would couple to any GPCR introduced into the system. Milligan and Rees have reviewed a group of modified G-protein subunits (referred to as chimeric G-proteins) that are modified at the C-terminus (the Gα coupling site to the receptor), to encourage “promiscuity” (i.e. the ability to interact with a large panel of GPCRs) in GPCR binding (Milligan & Rees 1999). These proteins are providing opportunities in cell-based and cell-free assays to monitor various GPCRs with the same reporter system.

Additionally, it would be worthwhile pursuing investigations into site-specific GPCR labelling using small organic fluorophores, a concept which has been investigated by a PhD student, Tamara Cooper, in our laboratory. This methodology, if further developed, may provide the ability to monitor conformational changes in the GPCR induced by ligand activation (as discussed in section 1.5.3), within a surface-bound vesicle, while avoiding the use of bulky fusion proteins. Use of the receptor alone would eliminate the steps required for G-protein purification and labelling prior to re-introduction into the system.

The future directions for research into the array platform most appropriate for the immobilization of GPCRs could involve further enrichment of the receptors within the lipid extract which make up the vesicles within the system. This could be investigated
using different techniques to produce native lipid vesicles (as discussed in section 4.1.2), or by developing generic, efficient purification protocols for GPCRs (as was investigated by a former PhD student in the lab, Amanda Aloia), which can be incorporated into pure lipid vesicle or cubosome systems. Studies involving the introduction of the complete signalling assembly (GPCR + G-proteins) and measurement of TR-FRET changes upon ligand addition on the oligo array surface, are still required. Additionally, further investigation is needed into the functional incorporation of proteins (specifically GPCRs and G-proteins) into the cubosomes particles discussed in chapter 4.

It is also worth noting the use of microfluidic devices (Whitesides 2006) in the application of GPCR array or sensors may provide a promising direction due to the use of small volume requirements, ideal for the small yields of precious receptor proteins such as those commonly obtained with GPCR expression.

5.4 Conclusion

The ability to create a homogeneous format to monitor GPCR activation can be achieved using TR-FRET. Homogeneous assay formats are more adaptable to substrate-based format as they require no separation or filtration steps. The ability to capture a functional GPCR onto a surface, specifically in a format adaptable to fluorescence microarray reading, is tackled with the use of native lipid vesicles carrying the receptors. Site-directed immobilization onto a microarray slide was achieved using complementary oligonucleotide tags. Alternative structures capable of providing a hydrophobic environment with potentially higher stability and protein capacity, known as cubosomes, were also capable of site-directed oligonucleotide immobilization.

Techniques such as TR-FRET and oligo directed native vesicle or cubosome immobilization are investigated for their adaptability in the development of array or sensor formats for GPCRs. While GPCR signalling has not yet been demonstrated using TR-FRET on an array platform, results in this thesis indicate that this strategy may be viable in the future.