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RESEARCH ARTICLE

G-protein coupled receptor array technologies: Site directed immobilisation of liposomes containing the H_1-histamine or M_2-muscarinic receptors

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This paper describes a novel strategy to create a microarray of G-protein coupled receptors (GPCRs), an important group of membrane proteins both physiologically and pharmacologically. The H_1-histamine receptor and the M_2-muscarinic receptor were both used as model GPCRs in this study. The receptor proteins were embedded in liposomes created from the cellular membrane extracts of *Spodoptera frugiperda* (*Sf9*) insect cell culture line with its accompanying baculovirus protein insert used for overexpression of the receptors. Once captured onto a surface these liposomes provide a favourable lipidic environment for the integral membrane proteins. Site directed immobilisation of these liposomes was achieved by introduction of cholesterol-modified oligonucleotides (oligos). These oligo/cholesterol conjugates incorporate within the lipid bilayer and were captured by the complementary oligo strand exposed on the surface. Sequence specific immobilisation was demonstrated using a quartz crystal microbalance with dissipation (QCM-D). Confirmatory results were also obtained by monitoring fluorescent ligand binding to GPCRs captured on a spotted oligo microarray using Confocal Laser Scanning Microscopy and the ZeptoREADER microarray imaging system. Sequence specific immobilisation of such biologically important membrane proteins could lead to the development of a heterogeneous self-sorting liposome array of GPCRs which would underpin a variety of future novel applications.

Keywords:
G-protein coupled receptors / Liposomes / Membrane protein microarray / Oligonucleotide directed immobilisation / Spotted arrays

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Abbreviations: [^{35}S]-GTPγS, radiolabeled isotope ^{35}S-conjugated to 5′-*O*-(3-thiophosphate); **bDNA,** biotinylated oligonucleotide; **BODIPY,** dipyrromethane boron difluoride; **cDNA,** cholesterol modified oligonucleotide; **GDP,** guanosine diphosphate; **GPCR,** G-protein coupled receptor; **GTP,** guanosine triphosphate; **H_1R,** H_1-histamine receptor; **M_2R,** M_2-muscarinic receptor; **NA,** neutravidin; **oligo,** oligonucleotide; **PEG-biotin,** biotinylated PEG; **PLL-*g*-PEG,** poly(L-lysine)-*grafted*-PEG; **QCM-D,** quartz crystal microbalance with dissipation; **SA532,** streptavidin Alexa fluor532; ***sf9,*** *Spodoptera frugiperda*; **TEM,** transmission electron microscopy

1 Introduction

G-protein coupled receptor (GPCRs) are cell surface receptors which have seven membrane spanning domains. They are the largest family of membrane proteins in the human genome and are involved in a number of physiological and pathophysiological pathways. They are the most widely targeted protein family for therapeutics being the target for over 40% of the currently available prescription drugs [1]. This is likely to increase with high-resolution structural data of such proteins now beginning to be published [2]. GPCRs bind to a wide variety of ligands ranging from volatile odorant mole-

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cules to biogenic amines and peptides. The list of known ligands to date is not comprehensive and there still exists more than 100 'orphan' receptors, to which the endogenous ligand is yet to be identified. The signalling pathway of the GPCR begins with a ligand binding event which usually occurs within the extracellular domain of the GPCR. This binding event activates the GPCR which in turn activates the coupled messengers termed G-proteins. The G-proteins consist of three dissimilar proteins, the Gα subunit and the Gβγ dimer. The G-proteins couple to the intracellular region (carboxy terminus) of the GPCR. Once the GPCR is activated the G-proteins undergo conformational changes which result in the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit. Once activated, the Gα and Gβγ dimer also alter their proximity to one another either by separation [3–5] or rearrangement [6].

There are a number of GPCRs that have been used as models for receptor immobilisation onto solid supports. These include the β_2-adrenergic receptor [7], the neurokinin-1 receptor (NK1R) [8], chemokine receptor CCR5 and CXCR4 [9], the human delta opioid receptor [10] and the α_{2a}-adrenergic receptor [11]. However in most instances, bovine rhodopsin has been used as it is one of the only GPCRs which has high natural abundance and for which the crystal structure is known [12–15].

There is substantial interest in establishing solid supports capable of functional immobilisation of membrane proteins such as GPCRs. One motivating factor is the belief that microarrays of membrane proteins would constitute a major advantage in the area of drug discovery by allowing parallel and high-throughput ligand screening. The extensive number of known and potential GPCRs encoded in the human genome together with the even larger number of potential ligands, establishes a basis for the advantages lying in such an approach (reviewed in ref. [16]). However, difficulties arise from the fact that proteins, in general, are chemically and structurally very diverse, often not very robust and subject to denaturation [17]. Membrane proteins, such as GPCRs, are particularly susceptible to denaturation because they are composed of large regions of hydrophobic residues, the structure of which, are destabilised in the aqueous environment. Due to these inherent microenvironmental requirements of membrane proteins, technologies establishing supported [18, 19] and free-spanning lipid bilayers [20–22], immobilised lipid vesicles [23–27], nanodiscs [28] or high-density lipoprotein particles [29] offer favourable interfaces to create arrays of membrane proteins. Many of these techniques, however, require the solubilisation and purification of the receptor prior to incorporation into prepared synthetic lipids. However, GPCRs are particularly difficult to isolate from their native membrane environment and the strategy of using crude membrane extracts might be advantageous.

Solubilised and purified receptors reconstituted into a lipid environment [7, 8] as well as receptors maintained in cell membrane extracts [30–33] 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 [7, 8, 12, 34], a histidine tag [29, 35] or GPCR-directed antibodies [7]. While evidence shows that in solution, receptors which are engineered with a tag for immobilisation retain G-protein coupling activity [29, 36, 37], there is little evidence to show G-protein activity whilst the receptors are attached to the surface, with the exception of surface plasmon resonance studies conducted using bovine rhodopsin [12].

Previous studies on which this work expands have demonstrated sequence specific and site-selective immobilisation of synthetic phospholipid vesicles onto a nonfouling and vesicle resistant PEG layer [23, 38]. Using complementary oligonucleotide (oligo) sequences, the vesicles have been captured on surfaces constructed using molecular assembly patterning by lift-off (MAPL) [38] or by spotting functional complexes [23]. While these studies have demonstrated the feasibility on model membranes and proteins, here we adapted the concept for the creation of functional GPCR arrays. In this study, *Spodoptera frugiperda* (*Sf*9) cell membrane extracts containing the H_1-histamine (H_1R) or M_2-muscarinic (M_2R) receptors have been prepared by differential centrifugation and attached to specific regions on a surface using complementary oligo sequences.

2 Materials and methods

2.1 Materials

Baculovirus stocks of G-protein subunits $G\alpha_{i1His}$ and $G\gamma_2$ were obtained from Prof. Richard Neubig, University of Michigan, USA, while $G\beta_4$ baculovirus stocks were obtained from Prof. James Garrison, University of Virginia, USA. GPCR baculovirus stock of the M_2R was obtained from Prof. Alfred Gilman, University of Texas, USA, and H_1-histamine receptor baculovirus was obtained from Prof. Willem J. DeGrip, University Medical Center, Nijmegen, Holland. Ratnala *et al.* [39] details further information regarding the generation of GPCR baculovirus stocks. ^{3}H-Scopolamine radioligand was purchased from Amersham Pharmacia Biotech. Radiolabeled isotope ^{35}S-conjugated to 5′-*O*-(3-thiophosphate) ([^{35}S]-GTPγS) and ^{3}H-pyrilamine were purchased from Perkin Elmer Life Sciences. Fluorescent ligands dipyrromethane boron difluoride (BODIPY)-pirenzepine (excitation/emission) 558/568 nm (Muscarinic antagonist) was purchased from Invitrogen. GTPγS and all other reagents (unless otherwise stated) of the highest quality grade were purchased from Sigma–Aldrich, Switzerland.

The graft copolymers poly(L-lysine)-*grafted*-PEG (PLL-*g*-PEG) and its biotinylated version PLL-*g*-PEG/biotinylated 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 PEG-biotin [3.4 kDa]. Oligo sequences were purchased from Eurogentec, Belgium and were as follows: bDNA1: 5′-CCC CCA TGG AAT CGT AA-3′; bDNA2: 5′-CCC CCT TCA GAG CAT AT-3′ both with a 5′ biotin modification; cholesterol modified oligo (cDNA)1: 5′-CCC CCT AGT TGT GAC GTA CAT TAC GAT TCC AT-3′; cDNA2: 5′-CCC CCT-AGT TGT GAC GTA CAA TAT GCT CTG AA-3′ both with a 5′ triethylene-glycol (TEG) cholesterol modification. Neutravidin (NA) and streptavidin Alexa fluor532 (SA532) were purchased from Invitrogen, Switzerland. In all cases, ultrapure water (Milli-Q Gradient, A 10 system, resistance of 18 MΩ/cm, total 4 ppb, Millipore Corporation, Switzerland) was used.

2.2 Cell culture, GPCR expression and preparation

*Sf*9 cells in SF900II media (Invitrogen, Australia) were grown in suspension culture at 28°C with shaking (138 rpm in an orbital shaker). Baculovirus stocks were filtered (using 0.2 μm syringe filter or by vacuum filtration using a Millipore Stericup) and added to *Sf*9 cells at 1–2 × 10^6 cells/mL to infect at a multiplicity of infection of 2. Infected cells were grown for 48–72 h at 28°C with shaking (138 rpm) prior to harvesting. *Sf*9 cell membranes containing either the H_1R or M_2R were prepared using a modified method to remove endogenously expressed G-proteins [40] as previously described [3].

2.3 Liposome preparation

*Sf*9 membrane preparations were resuspended to a total protein concentration of 0.75 mg/mL. Following this, some preparations were then extruded through a 1 μm polycarbonate membrane (Avestin, Germany) followed by a 400 nm polycarbonate membrane (Avestin) at room temperature. In some cases, this was followed again by a 200 nm polycarbonate membrane (Avestin). Total protein within the membrane extract was measured using the Bradford Protein Assay [41]. The membrane preparations were tagged with an oligo sequence by incubation (RT, 10 min) in a solution containing oligos modified with a cholesterol moiety (cDNA1 and cDNA2, see Section 2.1 for description) at a concentration of 0.35 μM.

2.4 Confirmation of GPCR expression and functionality

2.4.1 ^{3}H-Ligand binding

To determine receptor-ligand binding specificity, saturation curves were generated using ^{3}H-scopolamine for the M_2R preparation, and ^{3}H-pyrilamine for the H_1R preparation. Receptor preparations, at a total protein concentration of 1 μg, were incubated with various concentrations of the appropriate ^{3}H ligand in TMN buffer (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.6) in a final assay volume of 100 μL. To determine nonspecific binding, an additional concentration curve was generated in the presence of the appropriate receptor antagonists (100 μM Atropine or 100 μM triprolidine was used as antagonists for the M_2R and H_1R, respectively, to block the ^{3}H-ligand binding site). Specific binding was calculated by the subtraction of nonspecific binding from total binding. Samples were incubated for 90 min with gentle mixing at 27°C then filtered over glass microfiber filter papers (GF/C filters) (Filtech, Australia). Filters were washed three times with 4 mL of TMN buffer. Liquid Scintillant was added to the filters and a scintillation counter (Wallac 1410 liquid scintillation counter) was used to determine the amount of ^{3}H-ligand bound. K_d values for saturation curves were determined by nonlinear least-squares analysis fitted to a single hyperbolic binding function (GraphPad Prism V4.0, San Diego, CA, USA).

2.4.2 [^{35}S]-GTPγS binding assay

Functional activity of the receptors was measured by monitoring the activity of the second messenger G-proteins. Specifically, receptor induced activation of the Gα subunit was demonstrated using a radioactive GTP binding assay as previously described [3]. Purified $G\alpha_{i1}$ and $\beta_4\gamma_2$ G-protein subunits at a concentration of 20 nM were incubated with receptor membrane preparations. Agonist stimulated [^{35}S]-GTPγS binding studies were performed using a modification of published techniques [42]. A reconstitution mix consisting of 0.05 mg/mL M_2R or H_1R native membrane extracts, 5 μM GDP, 10 μM adenylylimidodiphosphate (AMP-PNP), 20 nM G-proteins and 0.25 nM [^{35}S]-GTPγS were prepared in a final volume of 100 μL in TMND buffer (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.6) on ice. The reactions were initiated with either buffer alone (basal) or in the presence of receptor agonist (1 mM histamine for the H_1R and 120 mM Carbachol for the M_2R). Specificity of agonist stimulation was determined by introducing a receptor antagonist (100 μM pyrilamine for the H_1R or 100 μM atropine for the M_2R) to block the agonist binding site and hence further signalling through the G-proteins. Samples were incubated for 90 min with gentle mixing at 27°C then filtered over GF/C filters. Filters were washed three times with 3 mL washes of TMN buffer. Liquid Scintillant was added to the filters and a scintillation counter (Wallac 1410 liquid scintillation counter) was used to determine the amount of [^{35}S]-GTPγS bound.

2.5 Electron microscopy

Membrane preparations were applied to lacey carbon grids which had been glow discharged under vacuum (30 s). 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 formed within the holes, during transmission electron microscopy (TEM). Excess solution was blotted for 10–30 s using filter paper. The grid was immediately plunged into liquid ethane to form a thin layer of vitreous ice containing the liposomes. 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 an FEI Technai 12 TEM operating at 120 kV and a magnification of 67 000 × or 110 000 ×.

2.6 Quartz crystal microbalance with dissipation studies

The quartz crystal microbalance with dissipation (QCM-D) instrument (Q-Sense E4, Sweden) was used to measure *in situ* changes in mass (measured by the frequency shift Δf) and viscoelasticity (measured by the dissipation factor, D) achieved through the adsorption of layers onto the surface of an oscillating crystal. The quartz crystal was coated with a 6 nm thin layer of Nb_2O_5 applied at the Paul Scherrer Institute (Switzerland). Prior to mounting in the liquid-exchange cell of the instrument, the substrates were cleaned by immersion in 2% w/w SDS for a minimum of 30 min, then rinsed in Milli-Q water and dried under a stream of nitrogen. This was followed by a UV/ozone treatment (30 min). Resonance frequencies were measured at several harmonic overtones (with overtone numbers of 3, 5 and 7). The surfaces were prepared in temperature stabilised (21–22°C) degassed HEPES buffer (10 mM 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid and 150 mM NaCl, pH 7.4) using modifications of published protocols [38]. A schematic of the surface modification process is shown in Fig. 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 functionalised and nonfunctionalised polymers as described previously [43]. If not stated otherwise, a mixture of 25% w/w PLL-*g*-PEG/PEG-biotin and 75% w/w nonfunctionalised PLL-*g*-PEG was used (12.5% of the PEG side chains biotinylated). Subsequently, the high affinity interaction of biotin and NA was utilised to tag the PEG-biotin chains with a biotinylated oligo (bDNA1 or bDNA2), 17 bases in length. The NA (0.33 μM) and biotinylated oligonucleotides (bDNA) (0.35 μM) were preincubated for 10 min in HEPES buffer prior to exposure to the biotinylated surface. Finally, liposomes (either extruded or nonextruded preparations) preincubated with a cholesterol modified oligo for at least 10 min (0.35 μM unless stated otherwise) in TMN buffer, were introduced into the QCM-D chamber. Prior to the injection of the GPCR membrane preparations, the buffer was exchanged with TMN. The Δf and ΔD for each modification step were monitored *in situ* and gave qualitative information on the mass and on the viscoelastic properties of the adsorbed layers, respectively.

2.7 Microarray preparation and imaging

Glass slides or Ta_2O_5 waveguides (Zeptosens, a division of Bayer (Schweiz) AG, Switzerland) were cleaned by ultrasonication for 10 min in 2-propanol, rinsed with ultrapure water, blow-dried with nitrogen, followed by an O_2 plasma treatment (2 min). Immediately after cleaning, the slides were coated with 100% w/w PLL-*g*-PEG/PEG-biotin (50% of the PEG chains biotinylated) by dipping the slides in the polymer solution (0.1 mg/mL, 40 min), followed by a rinse with Milli-Q water and blow-drying with nitrogen.

A pin and ring spotter (GMS 417 arrayer, Affymetrix, USA) was used for the contact printing of oligo spots

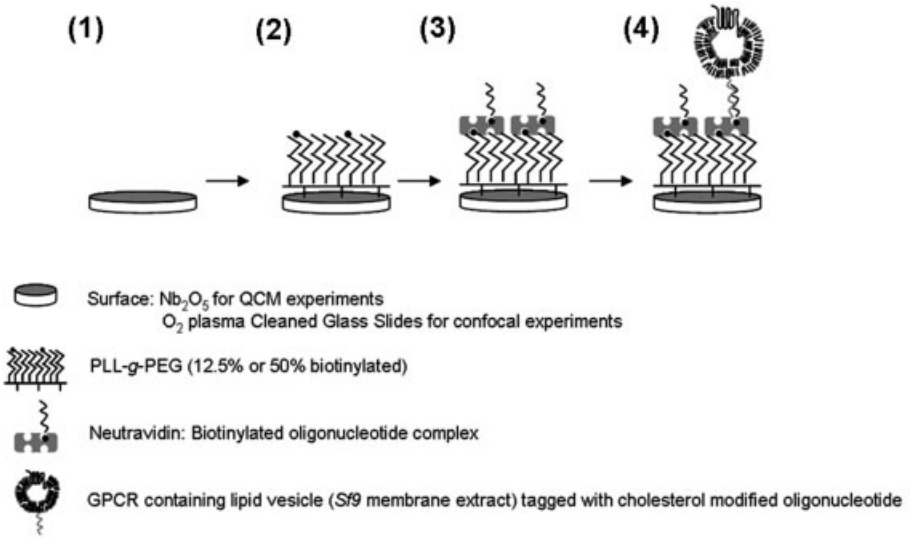

Figure 1. Schematic representation of the immobilisation protocol: (1) Clean surfaces were modified by (2) adsorption of PLL-*g*-PEG with 12.5 or 50% of the PEG chains terminated by biotin molecules (for QCM-D or array spotting, respectively). (3) Further modification included immobilisation of the preformed NA/bDNA complex. (4) Liposomes containing GPCRs that were tagged with complementary oligonucleotides modified with a cholesterol moiety were captured onto the oligo surface.

(~150 μm in diameter) according to a previously published protocol [23]. Briefly, the array was created by spotting complexes of NA (4.2 μM) and biotinylated oligos bDNA1 or bDNA2 (both at 4.4 μM) diluted in 50% zeptoMARK spotting buffer v/v (Zeptosens) onto the biotinylated polymer surface. Streptavidin Alexa fluor532 (2.5 μg/mL) was spotted as reference spots in the array.

Fluorescence experiments were performed in the zeptoCARRIER flow cell (Zeptosens). The slide was prewet with 200 μL TMN buffer after mounting. The membrane extracts were prepared as for the QCM-D experiment (Section 2.6) and added in a volume of at least 50 μL. After a 4 h incubation, the slides were rinsed twice with 200 μL buffer. The fluorescent GPCR ligand BODIPY-pirenzepine (muscarinic receptor) was used to monitor GPCR liposome immobilisation and added in the appropriate concentration after the membrane extract had been captured on the surface and unbound extract washed away. Fluorescent experiments were performed using either a Confocal Laser Scanning Microscope (ZeissLSM 510, Germany) or the ZeptoREADER (Zeptosens). The confocal microscopy images were taken with a diode pumped solid state (DPSS) laser (10 mW, 561 nm) using a 40 × (LD, NA 0.7) objective. Microarray imaging was performed with the ZeptoREADER (Zeptosens), a highly sensitive microarray reader based on waveguiding technology. Briefly, a laser light was coupled into and out from a Ta_2O_5 thin film deposited onto a glass substrate *via* an optical grating etched into the substrate. Surface-bound fluorophores detected by CCD camera were excited by the evanescent field resulting from total internal reflection of the laser beam making this technique extremely surface sensitive. The device was equipped with two lasers (532 and 635 nm) and a CCD camera for fluorescence detection, as further detailed in other publications [44–46].

3 Results

3.1 GPCR and G-protein expression and functionality

Sf9 cell expression of the M_2R and the H_1R was confirmed by the production of saturation binding curves using ^{3}H-scopolamine (Fig. 2a) and ^{3}H-pyrilamine (Fig. 2b), respectively. The ligand binding properties of the H_1R or the M_2R preparations did not change once the preparation had been extruded through a 400 nm polycarbonate filter (Fig. 2b). The apparent dissociation constant, K_d, for ^{3}H-scopolamine binding to the M_2R was 0.62 ± 0.075 nM, which is in a similar range to previously published results [47]. The K_d value for ^{3}H-pyrilamine binding to the H_1R was approximately 1.3 nM, which also agrees with published K_d results [48]. Saturation binding curves indicated that the M_2R had a maximum number of binding site (B_{max}) value of approximately 6.1 pmol/mg, similar to published expression levels [47] and the H_1R a value of 10.9 and 12.6 pmol/mg prior to and post extrusion, respectively (Fig. 2).

The membrane preparation carrying the H_1R (Fig. 3a) or M_2R (Fig. 3b) also retained its ability to couple to the heterotrimeric G-protein ($G\alpha_{i1}\beta_4\gamma_2$) once reconstituted in the [^{35}S]-GTPγS binding assay. Figure 3a shows the activity of the H_1R by displaying agonist (1 mM histamine) induced [^{35}S]-GTPγS binding to the receptor-associated $G\alpha_{i1}$ G-protein. [^{35}S]-GTPγS binding can be prevented by the introduction of an excess of antagonist (100 μM pyrilamine) to block the receptor's orthosteric agonist binding site. This is shown by the decrease of [^{35}S]-GTPγS binding to the level of basal activity (*i.e.* the level of binding that occurs when no agonist is present). The receptor activity is retained both prior to and post extrusion through 400 nm polycarbonate membranes. Similar results were obtained for the M_2R (Fig. 3b) using carbachol as agonist and atropine as antagonist.

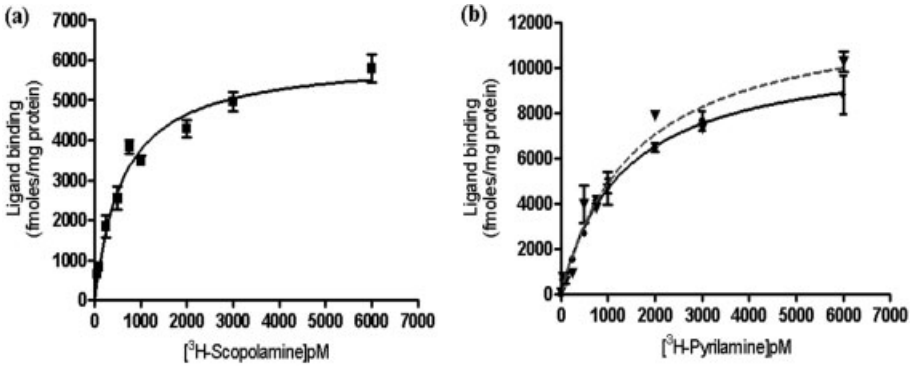

Figure 2. Specific binding of receptor ligands to *Sf9* membrane preparations. (a) (■) Specific M_2R binding (^{3}H-scopolamine) in *Sf9* membranes. Nonspecific binding was measured in the presence of 100 μM atropine (antagonist) and subtracted from total binding in the absence of the competitor (^{3}H-scopolamine); B_{max} = 6.1 ± 0.23 pmol/mg; K_d = 0.62 ± 0.075 nM, mean ± SEM, n = 5. (b) Specific H_1-histamine receptor binding (^{3}H-pyrilamine) in *Sf9* membranes. Nonspecific binding was measured in the presence of 10 μM triprolidine (antagonist) and subtracted from total binding in the absence of the competitor (^{3}H-pyrilamine). (●) Solid line, nonextruded membranes; B_{max} = 10.9 pmol/mg; K_d = 1.3 nM, n = 2; (▼) dotted line membrane preparation extruded through 400 nm polycarbonate membrane, B_{max} = 12.6 pmol/mg; K_d = 1.6 nM (n = 2, data shown is the mean and error bars represent the range of the duplicates).

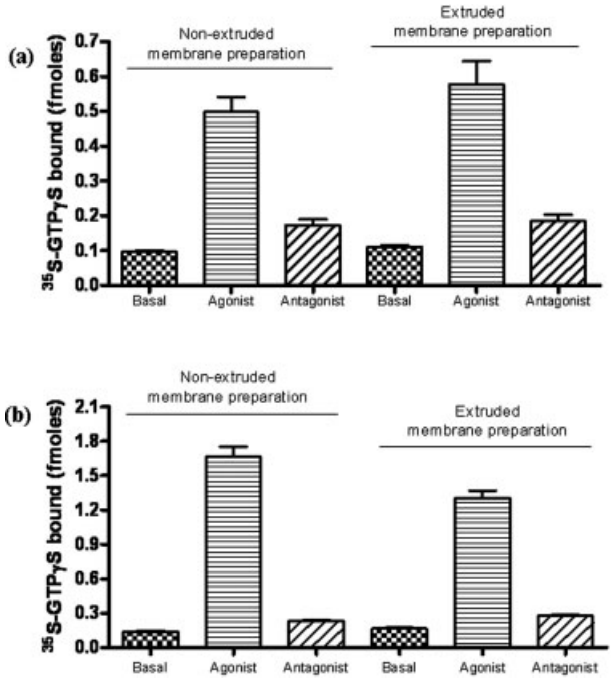

Figure 3. Assay of receptor signalling activity for reconstituted membranes containing M_2R or H_1R. 20 nM G-protein subunits, $G\alpha_{i1}$ and $G\beta_4\gamma_2$, were combined with 0.05 mg (total membrane protein)/mL receptor preparation; 0.25 nM [^{35}S]-GTPγS; 10 μM AMP-PNP; 5 μM GDP. This reconstitution mix was then incubated with either buffer alone (basal, black chequered column), an agonist (horizontal striped column) or an agonist in the presence of excess antagonist (diagonally striped column). (a) H_1R, stimulated with 1 mM histamine (agonist) and blocked with 100 μM pyrilamine (antagonist); (b) M_2R, stimulated with 120 mM carbachol (agonist) and blocked with 100 μM atropine (antagonist). Data shown are mean $\pm$ SEM ($n = 9$).

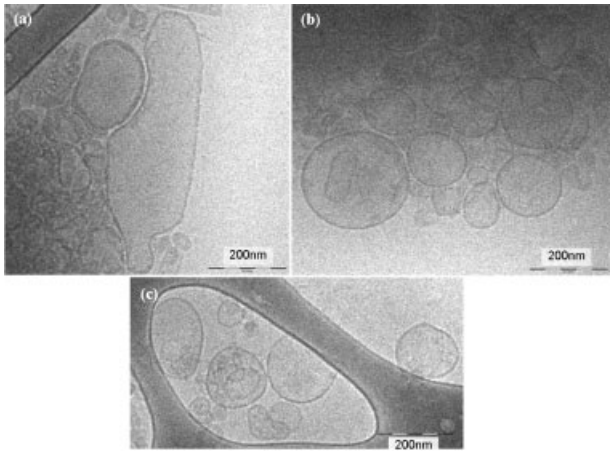

Figure 4. TEM images of liposomes: cryo-TEM images of crude *Sf9* cell membrane preparations containing the M_2R (a) and (b) nonextruded at 110 000 $\times$ magnification and 120 kV or (c) extruded through 400 nm polycarbonate membrane at 67 000 $\times$, in a thin layer of vitreous ice over a lacey carbon grid. Scale bars in all images represent 200 nm. Lacey carbon grids can be seen in dark grey in top left of image (a) and surrounding central vesicles in (c).

3.2 Electron microscopy

The membrane extracts contained liposome-type particles prior to extrusion (Figs. 4a and b). The solutions contained structures of various shapes and sizes (from 50 nm to $\geq$1 μm), with an outer layer approximately 5 nm wide. The size and shape of the structures which were generally spherical and fully enclosed indicated that the extracts were unilamellar liposomes. Once the preparation was extruded through a 400 nm polycarbonate filter, the liposomes appeared as a more homogeneous mix of spherical shapes and generally ranged in size from 50 to 400 nm (Fig. 4c).

3.3 Specific site-directed immobilisation of GPCR liposomes

3.3.1 QCM-D

The Nb_2O_5 surface of the quartz crystal was modified over a number of additions to the QCM-D flow cell. Frequency and dissipation shifts were monitored over each step (Figs. 5a and b). The first step involved the adsorption of the PLL-*g*-PEG/PEG-biotin onto the Nb_2O_5 surface. Surface saturation of the adsorbed PLL-*g*-PEG/PEG-biotin polymer was achieved within 40 min and resulted in a normalised change in the third frequency overtone ($\Delta f_3/3$) of -37 ± 1 Hz and a dissipation (ΔD_3) of $2.2 \pm 0.1 \times 10^{-6}$. The second step of the surface modification was the introduction of the NA/oligo complexes. NA/oligo complex binding to the biotinylated surface reached equilibrium within 1 h. The adsorption resulted in a $\Delta f_3/3 = -30.7 \pm 2$ Hz and $\Delta D = 3.0 \pm 0.3 \times 10^{-6}$. Both values are the mean $\pm$ SEM, $n = 10$ and are in agreement with previous reports [38]. Before injecting the liposomes, the buffer was exchanged from HEPES to TMN, a common assay buffer for the GPCR liposomes. This resulted in a small frequency change ($<$2 Hz) which stabilised before introduction of the GPCR liposomes.

The GPCR liposome preparations carrying the M_2R, of $<$400 nm in diameter were immobilised onto the surface within 1.5 h (Figs. 5a and b). A significant decrease in frequency (Fig. 5a) and an increase in dissipation (Fig. 5b) values indicated the successful immobilisation of GPCR liposomes carrying the oligo tag complementary to the one on the sensor surface. When the tag was present within the liposomes, the measured values were $\Delta f_3/3 = -208$ Hz and $\Delta D = 41 \times 10^{-6}$ (values are mean, $n = 2$). Surface immobilisation was oligo specific with low binding of untagged liposomes ($\Delta F_3/3 = -1.2$ Hz) (Figs. 5a and b) or liposomes carrying a noncomplementary oligo tag ($<$8 Hz) (data not shown). There was little nonspecific binding of liposomes to the surfaces at each step of the surface preparation (data not shown).

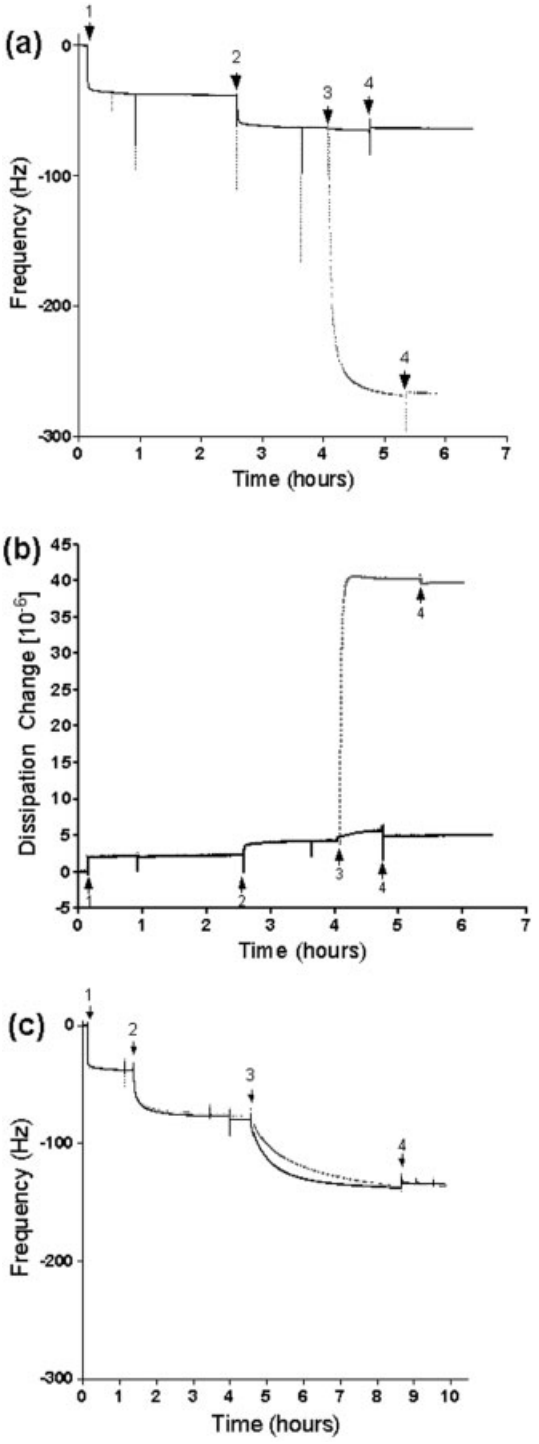

Figure 5. *Sf9* cell membrane liposome immobilisation. (a) Changes in frequency of overtone 3 and (b) changes in dissipation as measured on a QCM-D upon immobilisation of <200 nm liposomes composed of preparations of *Sf9* cell membranes containing overexpressed M_2R. (c) Changes in frequency of overtone 3 upon immobilisation of nonextruded extracts of *Sf9* cell membranes overexpressing M_2R (grey spotted line), H_1R (black solid line). The following steps refer to all graphs (a), (b) and (c) unless otherwise stated: (1) Adsorption of PLL-*g*-PEG (12.5% biotinylated) onto a Nb_2O_5 coated quartz crystal; (2) immobilisation of biotin-oligo complexed to NA; (3a) and (3b) surfaces are exposed to GPCR liposomes (extruded through 200 nm membranes) which carry no oligo sequence (black solid line), or to GPCR liposomes tagged with a complementary oligo sequence (grey spotted line); (3c) surfaces are exposed to non-extruded extracts tagged with complementary oligo sequence; (4) unbound liposomes are washed away with buffer. Unmarked peaks in the data sets indicate a buffer wash.

Specific liposome immobilisation was also achieved with H_1R and noninfected membrane extracts, and immobilisation was achieved for all three extracts in the extruded and nonextruded state. However, the total adsorbed mass for nonextruded liposomes varied from cell extract batch to batch and could be adjusted by varying the amount of cholesterol DNA added to the membrane extract preparations (results not shown). Furthermore, it was observed that for nonextruded liposomes, smaller changes in frequency and dissipation were observed and longer incubation times were required to reach binding saturation (>90% of the binding took place within 3 h), indicating

that there was a lower binding efficiency with these preparations (Fig. 5c).

3.3.2 Spotted arrays

GPCR liposomes were monitored using the fluorescent-labelled ligand BODIPY-pirenzepine (M_2R) (Fig. 6a). Sequence specific immobilisation of the GPCR liposomes onto the oligo arrays was further demonstrated using arrays composed of spots functionalised with two different oligo sequences (bDNA1 and bDNA2). Low nonspecific binding to the PEGylated background was observed. Spots functionalised with the noncomplementary oligo sequence displayed less than 20% of the fluorescence of the spots carrying the complementary oligo (Fig. 6b).

For the M_2R, ligand binding was found to be specific as shown by the zeptoREADER images of spots presenting either GPCR-free liposomes (Fig. 7a) or M_2R containing liposomes (Fig. 7b) after exposure to BODIPY-pirenzepine. However, as measured with the ZeptoREADER, 30% of the signal observed resulted from nonspecific ligand binding to the liposomes so that the signal measured for immobilised M_2R membrane preparations was about 3.3-fold higher compared to liposomes isolated from noninfected cells (pirenzepine concentration: 1.25 μM) (Fig. 7c). It should be noted that for this experiment, the immobilised liposome mass, measured in a control experiment performed with QCM-D, was similar.

4 Discussion

Results in this paper describe a method by which crude membrane preparations containing a specific membrane protein of interest, such as a GPCR, can be immobilised onto a specified site on a surface. This process is also conducive to enabling the immobilisation of a functional signalling complex consisting of the receptor and its second messengers, the G-proteins. This approach makes use of a standard oligo array which is converted into a heterogeneous membrane protein array, a strategy similar to that proposed by Niemeyer *et al*. [49–51] for a variety of biomolecules and nanoobjects. The following benefits of the methodology described should be highlighted: Firstly, nucleotide arrays have nowadays become a golden standard for several biological and biomedical applications [52, 53]; a great variety of production protocols have been proposed and several oligo arrays are commercially available for various biological and biomedical applications [52, 53]. Due to the large number of possible oligo sequences, it may be possible to specifically immobilise a large number of various membrane proteins in a microarray format. Furthermore, difficulties in the production of protein and membrane protein arrays which usually arise from the fact that proteins are chemically and structurally very diverse, less robust than oligonucleotides and generally subject to denaturation, may be overcome with this approach: the receptor's natural environment is maintained, and harmful drying steps are avoided, minimising the risk of functional alterations to the protein.

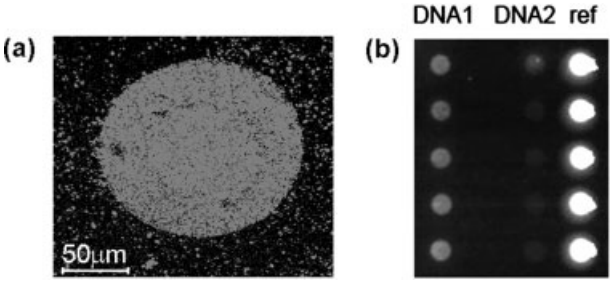

Figure 6. Site-specific immobilisation of liposomes containing GPCRs shown using (a) confocal microscopy image of oligo-tagged GPCR liposomes (M_2R) immobilised on a spot presenting the complementary oligo cDNA1 after incubation with BODIPY-pirenzepine (100 μM). (b) ZeptoREADER image of M_2R membrane extract tagged with cDNA1 attached to an oligo array presenting the oligo sequence bDNA1 and the oligo sequence bDNA2. ref represents an SA532 reference row (2.5 μg/mL).

This immobilisation strategy was shown to be well suited for the immobilisation of membrane extracts from noninfected cells as well as from cells overexpressing different GPCRs while preserving ligand binding specificity. This illustrates the flexibility and versatility of our approach. Furthermore, both extruded and nonextruded extracts could be immobilised with high-specificity as confirmed by QCM-D measurements.

The small batch-to-batch variations observed in the liposome binding properties measured by QCM-D could be the result of different DNA *per* liposome numbers for each batch which could lead to different binding efficiencies or liposome flattening as previously observed with synthetic vesicles [38], or different cholesterol incorporation efficiencies due to slight variation of the lipid composition between infected and noninfected *Sf*9 cells [54]. The presence of polysaccharides on the cell membrane surface with limited permeability [55] could also affect DNA hybridisation and explain differences in binding efficiency between extruded and nonextruded extracts. Preliminary results showed that the mass binding to the QCM sensor could be influenced by varying the concentration of cDNA added to the membrane extracts.

The membrane extracts were extruded in an attempt to homogenise the solution and minimise the variability that might be associated with a very heterogeneous mixture of liposome sizes. Characterisation of the extruded preparations using cryo-TEM indicated predominantly spherical, enclosed, unilamellar structures of sizes 50–400 nm in diameter. Images of nonextruded preparations, despite showing greater variation in size and shape, indicated that the extracts prior to extrusion, were also composed of liposomes. Nevertheless, to ensure maintenance of all components within the membrane necessary for native receptor function,

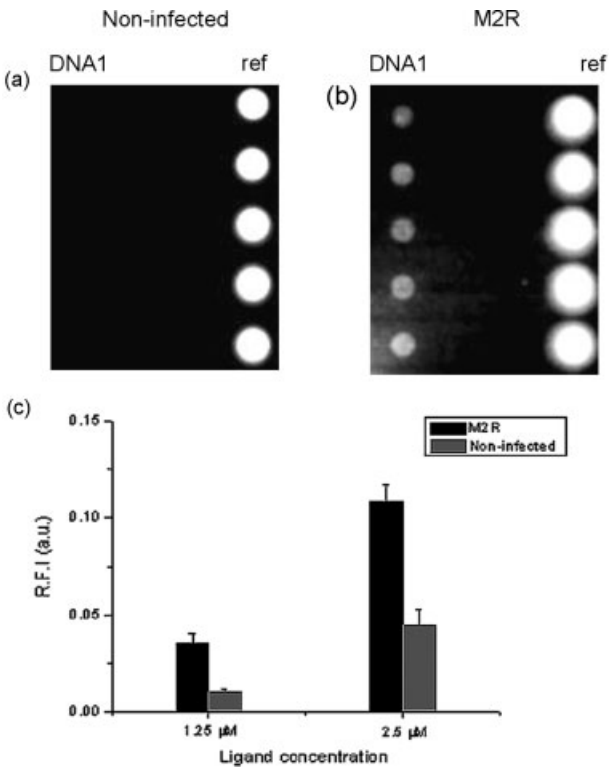

Figure 7. Fluorescent ligand binding to liposomes containing the M_2R. ZeptoREADER images show liposomes composed of native *Sf9* lipids (a) not expressing the M_2R, and (b) overexpressing the M_2R receptor captured *via* a complementary DNA pair. (c) Data obtained from a ZeptoREADER image of sequence specific oligo attachment. Fluorescence intensity normalised to the reference (reference is an SA532 reference row (2.5 μg/mL)) resulting from the binding of BODIPY-pirenzepine for two ligand concentrations. Black columns are the M_2R containing liposomes and the grey columns the noninfected liposomes.

and to minimise steps within the immobilisation protocol, membrane extracts which had not been extruded were used for work with the spotted arrays shown in this paper.

The use of crude membrane extracts without solubilisation and purification techniques results in limited control over issues such as the orientation of the receptor proteins within the liposomes. However the [^{35}S]-GTPγS binding assay indicates that the receptor protein orientation may not be a limiting factor. Results indicate a degree of 'leakiness' of these liposomes as both the intracellular carboxy terminus (G-protein binding site) and extracellular amino terminus (involved in ligand binding) must be accessible to enable both the agonist and [^{35}S]-GTPγS to bind. It is worth noting that most studies involving H_1R signalling, report of interactions with the $G\alpha_q$ subunit, however, in this *in vitro* assay system it was also shown to signal through the $G\alpha_{i1}$ subunit. We have not determined the relevance of this interaction with *in vivo* signalling as it is not within the scope of this paper, however, it has previously been reported that a functional H_1R-$G\alpha_{i1}$ interaction does occur *in vivo* [56]. The $G\alpha_{i1}$ subunit was used in this study because it binds [^{35}S]-GTPγS readily and specifically in this assay system.

In this paper, a single cholesterol moiety was used to anchor the liposome 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 DNA exchange between liposomes has been reported previously [26]. Therefore the method presented here enables only for the creation of heterogeneous arrays by a sequential immobilisation of the different liposome populations. An answer to this problem can potentially be found in the work of Pfeiffer and Höök [26] who have demonstrated that multiple cholesterol anchoring can provide a much stronger bilayer anchoring to the bilayer. Although not shown in this paper, preliminary data indicated that this double cholesterol/oligo tagging of the liposomes also resulted in the successful immobilisation of the GPCR containing liposomes onto a complementary surface. For mammalian cells, covalent coupling to azido groups could also provide an alternative [57].

Array images indicate some (20%) nonspecific ligand adsorption to the control spots with noncomplementary oligo strands (Fig. 6). Furthermore, nonspecific ligand binding (30%) to the liposomes regardless of receptor presence (Fig. 7) when the fluorescent pirenzepine was present at micromolar concentrations was also observed. A high concentration range was primarily used to ensure complete saturation of the receptors within the liposomes and to ensure clear visualisation. Higher concentrations were also used because pirenzepine has a lower affinity for the M_2 subtype of the muscarinic receptor family (K_i [equilibrium dissociation constant] = 303 nM) than it does for the M_1 subtype (K_i = 7.1 nM) [47]. This nonspecific binding indicates that the BODIPY-pirenzepine conjugate could be lipophilic, a feature of fluorescent conjugates of GPCR ligands that has been described previously [58, 59]. ZeptoREADER results, using fluorescent ligand concentrations in the lower micromolar range (2.5 and 1.25 μM) displayed approximately four times lower nonspecific binding on liposomes that did not contain GPCRs than images obtained using 100 μM of the fluorescent ligand (data not shown). Although these nonspecific binding effects do currently limit the sensitivity of the assay presented here, the specific signal was high enough to ensure accurate signal quantification upon normalisation of the data.

Furthermore, we have shown that this strategy provides the opportunity to immobilise a GPCR without the need to modify the receptor itself or to solubilise it from its native environment. The ability for the GPCR to retain its G-protein coupling capability may enable the production of a GPCR array which is less dependent on monitoring fluorescent ligand binding but relies more on true functionality. Limitations which lie in arrays reliant on fluorescent ligands 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 efficacy. An array format such as this which allows interaction freedoms for the receptor, including the ability to bind to various binding partners and undergo required conformational changes consistent with their physiological response, may provide the opportunity to monitor ligand activation through changes in associated proteins, such as G-protein conformational changes. This could be achieved by using a technique such as fluorescence resonance energy transfer (FRET) [60].

To conclude, we have demonstrated the use of surface immobilised oligos as a method of capturing membrane proteins. The liposomes used in the study were composed of native *Sf*9 cell membranes. These liposomes contained GPCRs expressed using the baculovirus/*Sf*9 expression system. The receptors were functional as demonstrated using ligand binding and receptor/G-protein signalling assays. These assays also indicate that both the intra- and extracellular domains of the receptor are available for small molecule binding. During surface immobilisation studies, the PLL-*g*-PEG polymer background showed little non-specific binding of these crude membrane preparations. QCM-D data and fluorescent array images captured by confocal microscopy and the zeptoREADER microarray scanner, demonstrate that capture of these liposomes occurs in a manner dependent on the oligo complementarities between those tagging the liposomes and those displayed on the surface. Such technologies will be useful in the field of membrane proteins, especially these proteins which are difficult to isolate from their native environment such as GPCRs.

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