Application of fragment-based drug discovery to membrane proteins

Früh, V.

Chapter

3

Functional immobilization of histamine H_1 and adenosine A_1 receptors on sepharose beads:

A facile approach with broad applicability to membrane proteins

G protein-coupled receptors (GPCRs) are responsible for signal transduction across cell membranes and are involved in many pathologies. Emerging new biophysical techniques can potentially provide more detailed information on protein-ligand interactions at the atomic scale. However, many of these technologies require protein immobilization, which remains a challenge when applied to GPCRs due to the absence of a generally applicable procedure. Here we address this issue by developing a simple and widely applicable immobilization protocol and applying it to crude membrane preparations containing either the human histamine H_1 receptor (hH_1R) or the human adenosine A_1 receptor (hA_1R). Native G proteins involved in the signalling cascade are retained during this process due to immobilization of crude, non-solubilized membrane fractions. The immobilization is based on Schiff's base formation between aldehyde groups on the resin and primary amines present in membrane-spanning proteins. Radioligand binding assays and dot blots show that this methodology succeeds in consistently yielding between 1 to 2 pmol of functional receptor per ml of resin. Pharmacological characterisation indicates that both antagonists and inverse agonists have similar affinity for immobilized and non-immobilized receptors suggesting the approach should be sufficiently reliable to carry out analytical assays for ligand discovery and characterisation. Furthermore, receptor immobilization results in significant stabilization and therefore the ability to store them. Thus, the method is promising as a means to immobilize a wide range of membrane proteins, including GPCRs without prior modification, solubilization, or lipid reconstitution.

G protein-coupled receptors (GPCRs) are responsible for transducing sensory and chemical signals across the cell membrane, and as such, their involvement in a wide range of pathologies makes them important drug targets and study foci. It is reported that approximately 30 - 40 % of marketable drugs target this class of proteins[100]. The two model receptors used in the scope of this study are prime drug targets since the histamine H_1 receptor mediates a variety of allergic reactions[183] and the adenosine A_1 receptor is involved in neurotransmission and thus a variety of neurodegenerative diseases[84]. Most GPCRs are ligand activated, yet a substantial number remain "orphaned", where the native activating ligand is not known. Discovery of non-native, small molecule modulators of GPCR function is also an area of highly active research for which gaps remain in current technologies. These issues underline the need to improve our understanding of this group of proteins by developing tools that provide new information in an efficient and detailed manner.

High throughput ligand screening assays of GPCRs typically use membrane-based assays involving microprinting[66,112,139] or rely on cell-based assays[184,185,186], where as many as 150,000 compounds can be screened in 8 hours. These assays are good at finding "drug-like" (300-500 Da) modulators of GPCR function, along with "macroscopic" parameters such as IC_{50} values, as well as some biological functionality characterizing the ligand-GPCR interaction. For an alternative and more "microscopic" approach, however, new biophysical methods are being developed in which the atomic or molecular interactions between a ligand and a protein are emphasized. Biophysical methods present many advantages such as the ability to directly detect physical interactions and differentiate between reversible and non-reversible processes. Furthermore, known functionality of the target protein is not required. Due to their intrinsic sensitivity to weak intermolecular interactions, many biophysical methods can be used to screen small molecule libraries of so-called drug fragments (150 - 300 Da)[22] (that obey Lipinski's rules)[10]. As a result, subsequent stages of the drug discovery process should yield compounds that are more orally bioavailable and less toxic.

Although biophysical techniques for high throughput screening have been successfully applied to soluble proteins that have been immobilized via a multitude of chemical linkers[101,102,103], applications to immobilized GPCRs still pose a challenge. GPCR conformation, stability, and

functionality are all dictated by experimental conditions ranging from cell culture and storage to the composition of the necessary presence of the lipid membrane[148]. In addition to this sensitivity, it is difficult to immobilize GPCRs with sufficient density to allow reliable signal detection. Many immobilization strategies employ protein modification by biotinylation[66] or the adjunction of antibodies[162] to a surface in order to have a well defined target orientation. Functional GPCR immobilization is commonly achieved by adsorption[137,187] or anchorage[146] of lipids on flat glass or gold surfaces, and are typically applied to purified, solubilized preparations which undergo subsequent lipid reconstitution[108,162,173] (Chapter 2). Schiff's base chemistry has been used in liquid chromatography as an immobilization strategy, but it has been reported to result in high non-specific binding[188]. Solubilization and lipid reconstitution of GPCRs require specific protocols for each protein, and finding the correct mixture of detergents can be extremely time consuming[189] or even futile. Furthermore, solubilization and purification results in the removal of the native membrane and associated proteins, such as the appropriate G proteins, which are important players in the signalling cascades[85,190]. A generally applicable method to immobilize GPCRs within their native membrane would clearly be welcome.

The aim of this study, therefore, is to determine whether GPCRs, as exemplified by the hH_1R and the hA_1R receptors, remain functional when they are immobilized in their native membranes on sepharose beads without modification, purification, or lipid reconstitution (Figure 1). This would provide a ready alternative to gold or glass chip surfaces used in current research and would allow GPCRs to be studied by a variety of biophysical methods such as SPR[170] and Target Immobilized NMR[23,77].

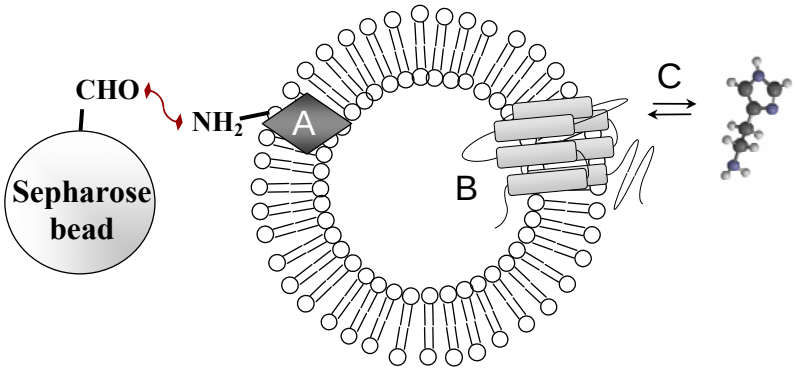

Figure 1. Immobilization of GPCRs on sepharose resin via native membrane vesicles. The immobilization is carried out *via* primary amines of the GPCRs (B) or of other integral or membrane associated proteins (A). The ligand binding properties (C) of such a system are essentially unchanged with respect to non-immobilized vesicles.

MATERIALS AND METHODS

Materials

The construction of the Chinese Hamster Ovary (CHO) stable cell line overexpressing the human H_1 receptor doubly tagged with heamaglutinin and 6-His (HA-hH_1R-HIS) will be reported elsewhere. The CHO stable cell line overexpressing the human A_1 receptor (hA_1R) was provided by Prof. Steve Hill at the University of Nottingham. HA-hH_1R-HIS and hA_1R had a maximum amount of binding sites (B_{max}) of 6.2 pmol/mg of total protein and 8.5 pmol/mg of total protein, respectively. All cell culture products such as Dulbecco's modified Eagle Medium, Penicillin, Streptomycin, Newborn bovine serum, G418, and Trypsin were purchased from standard suppliers. ALD Actigel coupling resin and coupling reagent $NaCNBH_3$ (sodium cyanoborohydride) were purchased from Sterogene (CA, USA). CH Sepharose 4B and NHS activated Sepharose 4 FF were purchased from GE Healthcare. [^{3}H]mepyramine (specific activity 32 Ci/mmol) was purchased from Amersham Biosciences (Roosendaal, NL) and [^{3}H]DPCPX (specific activity 127 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's-Hertogenbosch, NL). Histamine and mianserin, as well as all chemicals used for buffer preparations, were obtained from Sigma-Aldrich (Zwijndrecht, NL). CPA, 8-CPT, and N0840 were purchased from RBI (Natick, MA, U.S.A) and ADA (adenosine deaminase) was acquired from Roche Biochemicals (Mannheim, Germany). The antibody recognising $G\alpha_{q/11}$ proteins (sc-392) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell culture

Cells were cultured weekly in 30 Petri dishes (15 cm) with 20 ml modified Eagle's medium containing 50 IU/ml penicillin, 50 µg/ml streptomycin, 10 % (v/v) newborn bovine serum, and 400 µg/ml G418, at 37°C in humidified 5 % CO_2.

Membrane isolation and preparation

Weekly, cells were harvested and prepared according to the method described previously[191], with minor modifications to create a finer suspension. Cells were rinsed with PBS, detached by scraping, and washed by centrifugation at 2700 rpm for 5 min, with resuspension in 30 ml cold membrane buffer (15 mM Tris, 1 mM EGTA, 0.3 mM EDTA, 2 mM $MgCl_2$, pH 7.4 at 4°C). Cells were then homogenized in a tight-fitting 30 ml Potter-Elvehjem tube with 10 slow up and down strokes at 700 rpm (pottering), and immediately centrifuged at 1000 rpm for 10 minutes to remove unwanted pelleted cell debris. The supernatant was collected, pottered, and precipitated *via* ultracentrifugation at 31000 rpm for 20 minutes. The pellet was resuspended in 20 ml cold membrane buffer, and the pottering and ultracentrifugation steps were repeated. The resulting pellet was resuspended in 4 ml cold phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2HPO_4; pH 7.4 at 4 °C). For hA_1R containing membranes, an additional 0.8 IU/ml ADA was added to the final preparation. Total protein concentration was determined using the bicinchoninic acid (BCA) assay[192] before storing membranes at -20 °C.

Membrane immobilization

The ALD Actigel resin was used as a 50 % slurry. When possible, all procedures were carried out at 4 °C. The resin was first washed with filtered water prior to being washed 3 x with cold phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2HPO_4; pH 7.6 at 4 °C) by centrifugation at 3000 rpm and resuspended in an equal volume of the same buffer. Membranes were thawed and pottered with 5 strokes before being added to the Actigel resin at a ratio of 1 mg total protein to 1 ml of resin. Coupling reagent (1 M $NaCNBH_3$) was added to a final concentration of 0.1 M. The tubes were placed at 4 °C and gently rotated for 18 h so as to avoid pellet formation. After immobilization, the supernatant was collected for quantification after a 5 minute centrifugation at 3000 rpm. The pelleted resin was resuspended with 1 ml Tris buffer (100 mM Tris; pH 7.6 at 4ºC) and 0.1 M coupling reagent for 2h at room temperature to block the remainder of the free aldehyde sites on the resin. Reducing agent was always removed prior to continuing with

experiments by washing the resin four times with Tris buffer (50 mM Tris; pH 7.4 at 4 °C). To determine the effect of linker length on GPCR immobilization, a similar procedure was followed using either activated CH Sepharose 4B which has an 8 atom linker or NHS activated Sepharose 4 FF which has a 14 atom linker. Membrane vesicle preparations were thawed and immobilized according to the manufacturer's suggestions for each of the three resins using 1 mg total protein per 1 ml of resin. Immediately before radioligand binding assays, the resin was washed 4 times with cold Tris buffer. The amount of functional receptors immobilized was quantitated by radioligand binding studies as described below.

Quantitation of total receptor immobilization efficiency

To obtain an approximate quantification of the total amount of HA-hH_1R-HIS or hA_1R immobilized, dot blots were used with anti-HIS tag or anti-A_1R antibodies. 10 µl of fresh membrane preparations overexpressing the receptors were used as reference, while equivalent preparations from the non-recombinant, parental cell line membrane preparations not overexpressing receptors ($CHOK_1$ for hH_1R and CHOkool for hA_1R) were blotted as negative controls. Corresponding volumes of supernatants before and after the immobilization procedure were also blotted. The pelleted resin containing immobilized receptors were also sampled for blotting, after a 1:1 dilution in denaturing buffer used in SDS-PAGE analyses, but lacking bromophenol blue (50 mM Tris/HCl pH 6.8, 1 % SDS, 15 % glycerol, 1 % β-mercaptoethanol at room temperature) and a 15 minute incubation at 95 °C for removal of protein from the resin. After a 3 minute centrifugation at 2500 rpm, the supernatant containing the membrane vesicles stripped off the resin, along with denatured receptors, was blotted. All samples went through the denaturing step in order to compare results, and each lane consists of a serial dilution by a factor of 0.5. The bands were quantified by volume density analysis. The background level from the negative control was subtracted from the final values which were extrapolated by comparing the standard curve obtained with the known B_{max} and density analysis from the positive control.

Detection of $G\alpha_{q/11}$ proteins present with immobilized receptors on resin

Western Blot analysis of whole cell lysates, isolated membranes, and immobilized receptors was carried out to identify the presence of native $G\alpha_{q/11}$ proteins in the immobilized receptor's environment. Whole cell lysates and isolated membranes were used as positive controls and prepared in equivalent volumes for direct comparison with immobilized receptors. To prepare whole cell lysates, densely cultured cells overexpressing the HA-hH_1R-HIS were washed 3 times with PBS and scraped into tubes on ice before being centrifuged at 4000 rpm for 5 min at 4 °C. Cells were then resuspended in RIPA buffer (150 mM NaCl, 1 % NP-40, 0.1 % SDS, 2 µg/ml aprotinin, 5 µM leupeptin, 50 mM Tris; pH 8.0) and incubated for an hour at 4 °C before being pottered. Membranes were isolated and immobilized as described previously, but with the presence of 2 µg/ml aprotinin and 5 µM leupeptin. The pelleted resin containing immobilized receptors was washed 3 x to remove non-immobilized material and incubated for 15 minutes at 95 °C to melt the agarose resin, leading to collection of protein which was bound to the resin in the supernatant. Samples were centrifuged at 3000 rpm for 3 minutes and the supernatant was diluted 1:1 in loading buffer. Whole cell lysates and isolated membranes were diluted 1:1 and 1:2 in loading buffer. All samples were loaded at 10 µl, separated by 12 % SDS-PAGE and blotted onto a polvinylidene difluoride membrane before being detected by an enhanced chemiluminescence assay, with the $G\alpha_{q/11}$ protein antibody as a primary antibody. The bands were quantified by volume density analysis with subtraction of the background levels from the negative control, extrapolated values from the standard curve obtained with the known B_{max} and density analysis from the positive control containing 1:1 cell lysates.

Pharmacological characterisation of non-immobilized and immobilized HA-hH_1R-HIS receptors and hA_1R receptors

Displacement studies were carried out by incubating HA-hH_1R-HIS membrane aliquots of 5 µg total protein for 30 min at 30 °C in 400 µl Tris buffer (50 mM Tris; pH 7.4 at 4 °C) containing final concentrations of 1 nM [^{3}H]mepyramine and increasing concentrations of displacer, either

mianserin (antagonist) or histamine (agonist). Saturation studies were carried out by incubating HA-hH_1R-HIS membrane aliquots of 5 μg total protein with increasing concentrations of [^{3}H]mepyramine ranging from 0.1 nM to 8 nM with non-specific binding determined by the presence of 1 μM mianserin. The incubations were stopped by rapid dilution with ice-cold Tris buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters (Whatman, Belgium) that had been treated with 0.3 % polyethylenimine as described previously[193]. Filters were washed four times with binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. Binding studies for hA_1R were similar, however a 60 min incubation time at 25 °C was required with final concentrations of 1 nM [^{3}H]DPCPX with increasing concentrations of CPA (agonist), N0840 (antagonist), and 8-CPT (inverse agonist). Saturation curves were determined with increasing concentrations of [^{3}H]DPCPX ranging from 0.1 to 4 nM, using 10 μM CPA to determine non-specific binding. Filtration was done over Whatman GF/B filters without prior polyethylenimine treatment. Immobilized receptors were characterised in an identical manner to the respective non-immobilized receptors, by using an amount of resin which corresponded to 5 μg of total immobilized protein. All radioligand binding studies of membranes immobilized on resin required special attention because resin covered the filter surface and could easily be dispersed. Furthermore, the incubation steps were carried out without shaking to prevent loss of resin due to adherence on the edges of the tube.

In order to quantify the amount of functional receptor immobilization, samples were incubated with and without a displacer to determine saturation curves in the presence of the corresponding radioligand (1 nM). Appropriate controls were chosen to determine fmol of functional receptor present in the various steps. To determine the effect of the reducing agent on ligand binding, membranes were incubated with 0.1 M $NaCNBH_3$, which was subsequently removed by pelleting the membrane preparations and resuspending in Tris buffer. For stability studies, samples were kept for one week at 4 ºC and subjected to quantification as described above.

Data analysis

Receptor binding data were analysed using the non-linear regression curve fitting program Graph Pad Prism v. 4.01 (Graph Pad, San Diego, CA, U.S.A.). Statistical significance was evaluated with the student's T-test. Saturation experimental data (K_d and B_{max} values) were obtained by computer analysis of saturation curves. Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng & Prusoff equation $K_i=IC_{50}/(1+[C]/K_d)$, where [C] is the concentration of radioligand used in competition binding, and K_d its dissociation constant[194]. All values obtained are means of at least three independent experiments performed in duplicate. Values of functional receptor were derived by the following equation, assuming that each mole of radioligand binds to one mole of receptor: $R= [[C]/(K_d + [C])] \times [S/(2220 \times SP)]$ where R is the amount of functional receptors (moles) per 50 µl resin, [C] is the radioligand concentration, S is the radioligand specific binding (dpm), and SP is the radioligand specific activity (Ci/mmol). In all assays, care was taken to assure total binding never surpassed 10 % of total radioligand added. In order to determine how much total protein was immobilized, the amount of protein added to resin and the amount present in supernatants after immobilization were subject to BCA protein assays, after a wash with buffer and an ultracentrifugation step to remove $NaCNBH_3$ due to its negative effect on the assays. The volume density analysis of dot blots and western blots were carried out using Quantity One imaging software (BioRad, USA).

RESULTS

We sought an immobilization procedure that would both maintain the native environment and be widely applicable to an array of potential membrane protein targets, yet compatible with various biophysical assays. We therefore began by attempting to immobilize membrane preparations of cells stably expressing human GPCRs. We chose sepharose based resins that are well characterized for bio-compatibility, have low non-specific binding and are highly porous endowing large specific binding capacity. The Schiff's base chemistry used to immobilize proteins on commercially available sepharose resins is very mild, yet stable. Various membrane preparation methods were tried, but ultimately, that which resulted in a fine suspension by repeated pottering and centrifugation was used (see Methods). Using this method, a Heamaglutinin and 6-His tagged human H_1 receptor (HA-hH_1R-HIS) and untagged human A_1 receptor (hA_1R) were consistently immobilized on the Actigel ALD resin (Figure 2, panels **a** and **b** respectively).

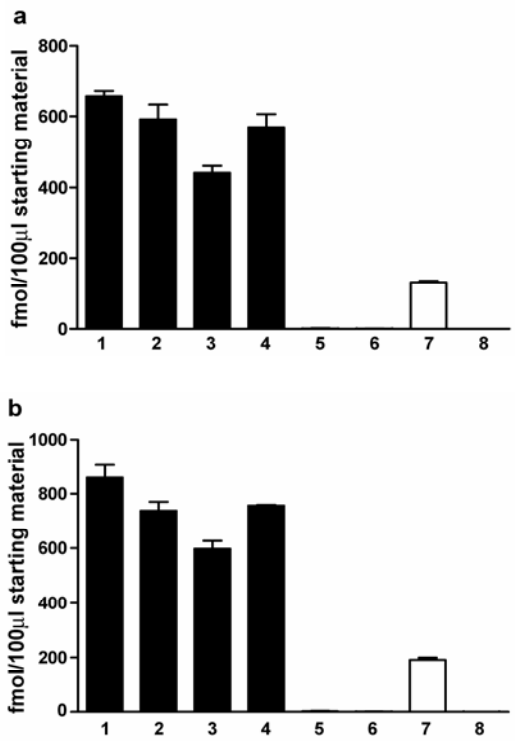

Figure 2. Immobilization efficiency of HA-hH_1R-HIS membranes (**a**) and hA_1R membranes (**b**) as measured by radioligand binding. The height of each bar represents the fmol of radioligand bound per 100 µl of starting material. Black bars represent controls of membrane preparations with the first being the starting material stored at -20 °C (1) and the second representing preparations that were maintained at 4 °C for the same duration as samples that were immobilized (2), the same as 2 but in the presence of reducing agent (3) and after removal of the reducing agent (4). Non-specific ligand binding to untreated resin was measured (5), along with the washing efficiency (6). The amount of functional, immobilized GPCR was determined (7) as well as that which remained in the supernatant after immobilization (8). The average of 3 experiments performed in duplicate is shown

The starting material consisted of membrane preparations that had been stored at -20 °C. We first titrated the amount of membrane preparation used for immobilization and observed a distinct optimum achieved when adding receptors at 1 mg total protein/ml concentration, at a maximum of 1 mg total protein per ml (settled bed volume) of resin (data not shown). Assuming 1:1 ligand binding stoicheometry, this amount corresponds to 620 fmol of active HA-hH_1R-HIS and 850 fmol of active hA_1R per 100 µl of starting material (Figure 2**a** and Figure 2**b**, column 1). Both receptors show consistency in the proportion of active receptors remaining at each step of this experiment. Simple storage of the membrane preparations at 4 ^{o}C for 18 h resulted in a 10 % loss of ligand binding capacity for both receptors (Figure 2**a** and Figure 2**b**, column 2). While the presence of the reducing agent sodium cyanoborohydride ($NaCNBH_3$, for reducing the Schiff's base to a primary amine) had a slightly negative impact on the radioligand binding assays, this was completely reversible (Figure 2**a** and Figure 2**b**, columns 3 & 4). There was little non-specific binding of the radioligand to the resin in the absence of receptor and the washing step was efficient in removing all receptors that were not irreversibly immobilized by $NaCNBH_3$ (Figure 2**a** and Figure 2**b**, columns 5 & 6). The mild procedure results in consistent functional immobilization of 20 - 25 % of both the hH_1R and hA_1R receptors. Essentially no detectable functional receptors remained in the supernatant fraction after immobilization for 18 h (Figure 2**a** and Figure 2**b**, columns 7 & 8). This equates to 1.3 pmol of functional HA-hH_1R-HIS and 2.1 pmol of functional hA_1R per ml of resin.

Since the radioligand binding assay could only detect functional receptors, we wished to know whether or not the coupling procedure was selecting for functional receptors leading to the apparent 20 - 25 % yield. Using a total protein quantification method, we found approximately 1 % of the input protein remaining in the supernatant after an 18 h immobilization (not shown). This data corresponds well to the amount of receptors in the supernatant after immobilization when determined by radioligand binding studies. We used a dot blot assay in order to specifically follow the fate of the HA-hH_1R-HIS during immobilization (Figure 3**a**).

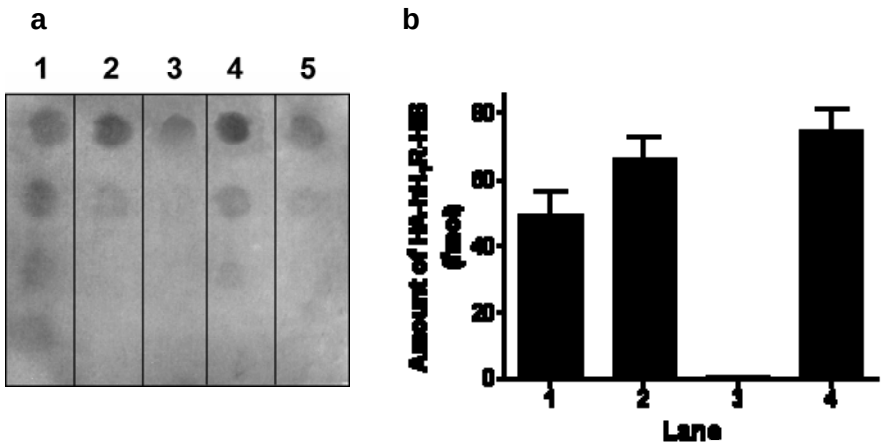

Figure 3. Immobilization efficiency of HA-hH_1R-HIS-containing vesicles. A dot blot assay was used to quantitate the amount of HA-hH_1R-HIS present in various fractions of the immobilization procedure using anti-HIS tag antibodies (**a**). A control vesicle preparation with approximately 6 fmol/µl of HA-hH_1R-HIS (lane 1) is compared to the supernatant of an immobilization reaction before (lane 2) and after immobilization (lane 3), from which it can be seen that the majority of receptor is removed from solution. Analysis of the supernatant after stripping the receptors off the beads (lane 4) indicates that the majority of receptors were tightly bound to the resin (lane 4). Vesicle preparations from the parental $CHOK_1$ cell line that does not express the receptor indicate the level of non-specific staining (lane 5). The dot volume densities were quantified by QuantityOne (BioRad) and the amount of HA-hH_1R-HIS in the first row of each lane is represented in (**b**) after subtraction of non-specific signal in lane 5.

The blots were quantified and the amount of receptor in the first row of each lane is represented in Figure 3**b** after subtraction of background signal from the negative control in lane 5. Fresh membranes stored at -20 ºC were blotted in (lane 1) and represent the positive control. Membrane suspensions before (lane 2) and after immobilization (lane 3) show that a significant fraction of the receptor has been removed from solution (compare lanes 2 and 3) and as expected, was bound to the resin (lane 4). The apparent increase in signal was due to concentration of the receptor sample upon immobilization which was difficult to precisely correct for due to the unknown efficiency with which the covalently bound protein could be removed from the resin by heating. The signal remaining in the post-immobilization supernatant was consistent with the amount observed with radioligand studies of the supernatant after immobilization, and is within the same level, within error, of the negative control membranes that do not express His-tagged receptors (lane 5). This data suggests that nearly all of the GPCRs have been successfully immobilized on the resin and that therefore, only 20 - 25 % of this population remained

functional as suggested by the data in Figure 1. Similar experiments were attempted for the hA_1R but dot blots were inconclusive as the anti-hA_1R antibodies apparently did not recognize denatured receptors.

The aim of immobilizing GPCRs via their native membrane was to keep as much of the native environment present as possible, specifically including all proteins necessary for signal transduction. Thus we sought to determine whether the appropriate G proteins for hH_1R, $G\alpha_{q/11}$, were co-immobilized on the sepharose beads. Western Blot analysis (Figure 4**a** and Figure 4**b**)

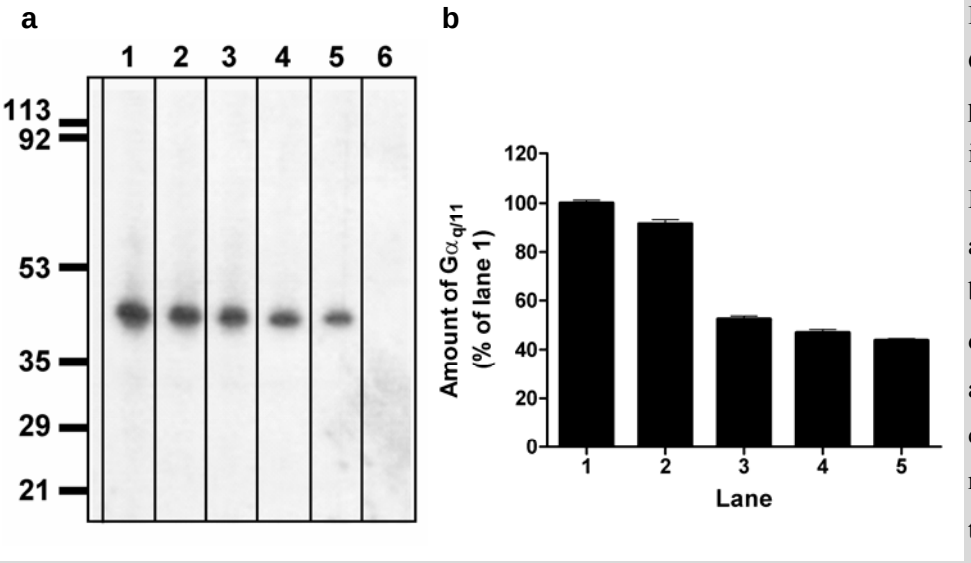

Figure 4. Detection of $G\alpha_{q/11}$ proteins (42 kDa) present on the resin with immobilized HA-hH_1R-HIS by Western Blot analysis. $G\alpha_{q/11}$ antibodies were used to detect $G\alpha_{q/11}$ proteins (**a**) and the corresponding quantities of $G\alpha_{q/11}$ are represented as percentages of lane 1 (**b**). Lanes 1 to 4 correspond to positive controls, where lanes 1 and 3 are lysates of whole CHO cells overexpressing the receptor, with no dilution and a dilution of ½ respectively, and lane 2 and 4 are the corresponding isolated membranes, with the same pattern of dilutions. Lane 5 corresponds to the supernatant containing membrane vesicles which have been denatured off the resin after immobilization, with no dilution. Lane 6 contains the standard proteins used as a ladder for the MW weights, and consists of a negative control due to the absence of $G\alpha_{q/11}$ proteins. The quantification reveals that 45 % of the proteins applied to the resin (lane 2) were collected from the resin after denaturation (lane 5).

show that the amount of $G\alpha_{q/11}$ proteins immobilized equates to 45 % of the amount present in HA-hH_1R-HIS whole cell lysates and isolated membranes. It was not our goal to quantify the exact amount of $G\alpha_{q/11}$ proteins which were co-immobilized, because the level of uncoupling from the resin achieved with our method is not fully quantifyable. Therefore, although it is

difficult to say whether there were more $G\alpha_{q/11}$ proteins immobilized but only 45 % were effectively stripped off the resin, this data suggests that the physiologically relevant $G\alpha_{q/11}$ proteins were co-immobilized on the resin and therefore enabled agonist binding pharmacology on the immobilized HA-hH_1R-HIS receptors.

We wanted to determine any possible influence of linker length on functional immobilization of receptors. The sepharose ALD has a relatively short 5 atom linker so we tested resins with hydrophilic linkers of 8 and 14 atoms for functional immobilization of hA_1R and HA-hH_1R-HIS receptors (Figure 5).

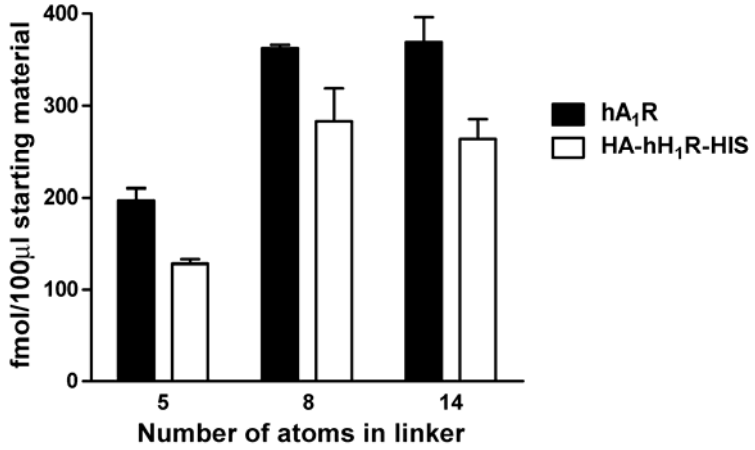

Figure 5. Determination of linker length on efficiency of functional immobilization of hA_1R and HA-hH_1R-HIS. The total amount of functional receptor bound was determined by saturation binding experiments. The average of 3 experiments performed in duplicate is shown.

Both resins resulted in a near doubling of the amount of functionally immobilized receptors. Since the immobilization chemistry of all three resins is very similar, it seems likely that there is a threshold of linker length required for maximal functionality. This data is consistent with the idea that the receptor is sterically hindered by shorter linkers[189].

To characterize the pharmacology of immobilized receptors, both saturation and competition binding studies were performed on non-immobilized and receptors immobilized on the ALD resin with a 5 atom linker. Values obtained from saturation studies (Figure 6) showed similar equilibrium dissociation constants (K_d) of [^{3}H]mepyramine for the non-immobilized and immobilized HA-hH$_1$R-HIS.

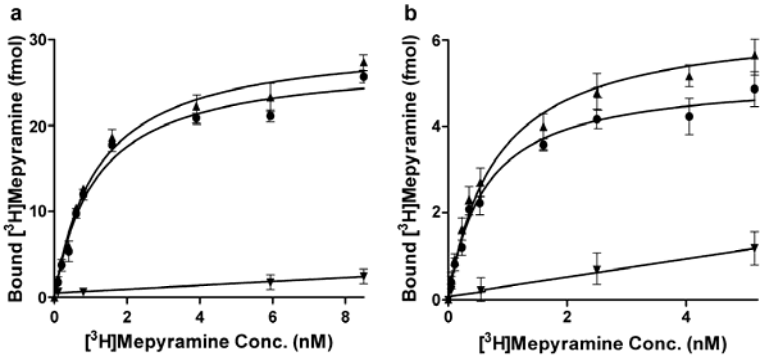

Figure 6. Saturation binding of [^{3}H]mepyramine to non-immobilized (**a**) and immobilized HA-hH1R-HIS (**b**). The amount of [^{3}H]mepyramine bound is indicated for the various fractions of total (▲), non-specific (▼),and specific binding (●). The average of 3 experiments performed in duplicate is shown.

As can be seen in Figure 2a, the immobilized receptor has a lower B_{max} (1.3 pmol/mg vs. 6.2 pmol/mg for non-immobilized, Table 1) while displaying a level of non-specific binding consistent to the levels found when the receptor was not coupled to the resin. Displacement studies were used to determine the binding constant of the agonist histamine and the antagonist mianserin on both non-immobilized and immobilized HA-hH$_1$R-HIS. In the case of histamine, both non-immobilized and immobilized receptor data were best fit by a 2-site model (Figure 7**a**, T-test; $p < 0.01$ and Figure 7**b**, T-test; $p < 0.01$, Table 1).

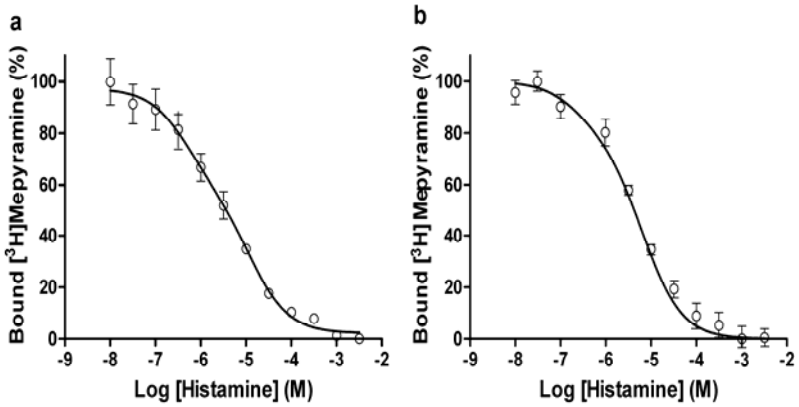

Figure 7. Displacement of specific [^{3}H]mepyramine binding to non-immobilized (**a**) and immobilized (**b**) HAhH$_1$R-HIS receptors by the agonist histamine. Both curves are best fit by a two-site model. The average of 3 experiments performed in duplicate is shown.

The affinity of the immobilized HA-hH_1R-HIS receptor for histamine was similar to the non immobilized receptor. Although there may be a slightly larger fraction of high affinity receptors in the immobilized sample (Table 1), it is not possible to distinguish a difference due to experimental uncertainty. hA_1R immobilized on the 5 atom linker ALD resin displayed the same pattern of ligand interaction as the HA-hH_1R-HIS receptor.

	N/I	Ligand	Binding state	Constant	B_{max}
A	N	[^{3}H]Mepyr-amine		K_d 1.1 ± 0.1 nM	6.2 ± 0.3 pmol/mg total protein
	I			K_d 0.7 ± 0.7 nM	1.3 ± 0.0 pmol/mg total protein
B	N	Histamine	2-site	K_L 20 ± 2 μM	
	N			K_H 0.4 ± 0.4 μM	
	N			R_H 55 ± 10 %	
	I			K_L 38 ± 6 μM	
	I			K_H 1 ± 2 μM	
	I			R_H 78 ± 24 %	
	N	Mianserin	1-site	K_i 0.3 ± 0.0 nM	
	I			K_i 0.6 ± 0.1 nM	

Table 1. Affinity and binding capacity estimates obtained from saturation binding curves (**A**) and competition binding curves (**B**) of non-immobilized (N) and immobilized (I) HA-hH_1R-HIS. Dissociation constants (K_d) and maximum amount of binding sites (B_{max}) were obtained from saturation curves, with 1 μM mianserin to determine non-specific binding. Competition binding constants of the agonist histamine and the antagonist mianserin provided low affinity constants (K_L), high affinity constants (K_H), and percentages of high affinity receptor populations (R_H) from 2-site binding curves and affinity constants (K_i) from 1-site binding curves.

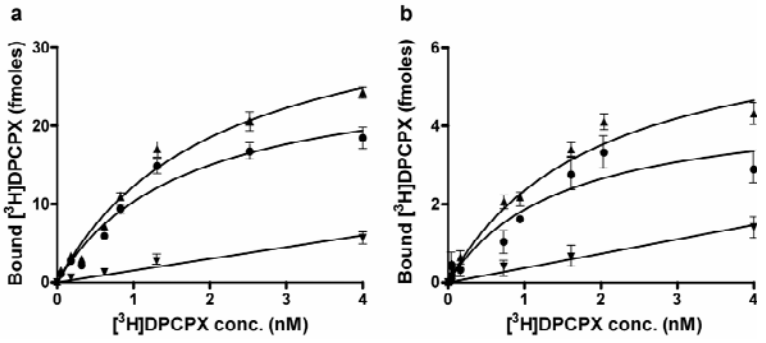

Figure 8. Saturation binding of [^{3}H]DPCPX to non-immobilized (**a**) and immobilized hA_1R (**b**) receptors. The amount of [^{3}H]DPCPX bound is indicated for the total (▲), non-specific (▼), and specific binding (●). The average of 3 experiments performed in duplicate is shown.

The saturation curve again indicated that approximately 25 % of the input receptor (relating to maximum functionality at 4 °C after 18 h incubation) had been functionally immobilized (Figure 8) and that non-specific binding, determined in the presence of 10 μM N^6-cyclopentyladenosine (CPA), was consistent with than for the non-immobilized preparation.

The equilibrium dissociation constant (K_d) of [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) for non-immobilized receptors was marginally larger than for immobilized ones, but still within experimental error (Table 2).

	N/I	Ligand	Binding state	Constant	Bmax
A	N	[3H]DPCPX		K_d 1.7 ± 0.7 nM	8.5 ± 0.8 pmol/mg total protein
	I			K_d 2.1 ± 0.4 nM	2.1 ± 0.2 pmol/mg total protein
B	N	CPA		K_L 250 ± 50 nM	
	N			K_H 3 ± 0 nM	
	N		2-site	R_H 55 ± 10 %	
	I			K_L 202 ± 100 nM	
	I			K_H 3 ± 2 nM	
	I			R_H 41 ± 7 %	
	N	8-CPT	1-site	K_i 28 ± 1 nM	
	I			K_i 39 ± 16 nM	
	N	N0840		K_i 440 ± 80 nM	
	I		1-site	K_i 540 ± 70 nM	

Table 2. Values obtained from saturation binding curves (**A**) and competition binding curves (**B**) of non-immobilized (N) and immobilized (I) hA_1R. Dissociation constants (K_d) and maximum amount of binding sites (Bmax) were obtained from saturation curves, with 10 μM CPA to determine non-specific binding. Competition binding constants of the agonist CPA, the inverse agonist 8-CPT, and the antagonist N0840 provided low affinity constants (K_L), high affinity constants (K_H), and percentages of high affinity receptor populations (R_H) from 2-site binding curves and affinity constants (K_i) from 1-site binding curves.

Agonist binding of CPA to non-immobilized and immobilized receptors was modelled on the basis of two different binding populations (Figure 9**a**, T-test, $p < 0.01$, Figure 9**b**, T-test, $p < 0.01$, Table 2). As for the HA-hH_1R-HIS receptor, the affinities of CPA for the receptors, whether immobilized or in solution, were very similar. In the case of the hA_1R receptor however, the

fraction of high affinity receptors is slightly less in the immobilized state than non-immobilized state and the difference is greater than the experimental error. The inverse agonist 8-cyclopentyl-1,3-dimethylxanthine (8-CPT) binds the immobilized receptor with slightly reduced affinity although again, within experimental error, while the antagonist N^6-cyclopentyl-9-methyladenine (N0840) displays lower affinity for the immobilized hA_1R than the non-immobilized receptor (Table 2).

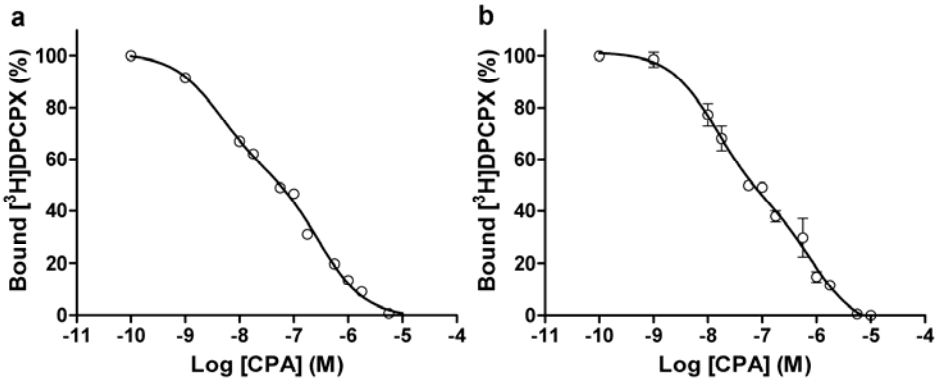

Figure 9. Displacement of specific [3H]DPCPX bin-ding to non-immobilized (**a**) and immobilized (**b**) hA1R receptors by the agonist CPA. Both curves are best fit by a two-site model. The average of 3 experiments performed in

Theory suggests that protein immobilization should result in stabilization due to decreased entropy of the unfolded state (from steric hindrance of the support). However, in the case of integral membrane proteins whose entropy in the unfolded state may be constrained by the membrane, it is not clear whether immobilization would have any effect. We investigated the effect of immobilization on GPCR stability by storing immobilized and non-immobilized preparation at 4 °C. After one week at 4 °C, both hA_1R and HA-hH_1R-HIS receptors maintained significantly higher activity when they were immobilized compared to when they were kept in solution (Figure 10).

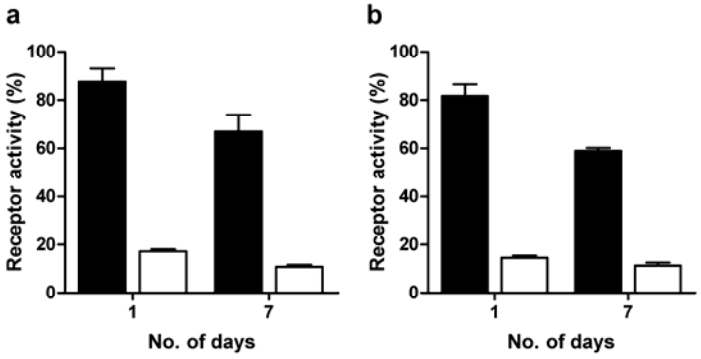

Figure 10. Stability of HA-hH_1R-HIS (**a**) and hA_1R (**b**) membranes as measured by radioligand binding and normalized to percentage on day 0. Membrane preparations, both immobilized and non-immobilized, were stored at 4 °C. Black bars represent immobilized membranes. White bars represent membrane preparations in solution.

DISCUSSION

Our results show that it is possible to immobilize functional GPCRs within their native membranes on sepharose beads without receptor solubilization, purification or lipid reconstitution. The method resulted in the co-immobilization of other membrane proteins, both integral and membrane associated with similar efficiency. Furthermore, receptors immobilized in this manner remained stable for up to a week at 4 ºC at least. The methodology applied was simple, repeatable, and consistently yielded up to 1 - 2 pmol of functional receptor per ml of resin with two different receptors. While we only used GPCRs in our work, it is reasonable to expect that a similar approach should also work for other integral and membrane associated proteins such as ion channels, chemokine receptors or cytochrome P450s.

A significant shortcoming of methods that rely on purification and solubilization is that upstream or downstream components of the signalling cascade may be lost upon immobilization. In the case of GPCRs, full functionality clearly requires the presence of other players within the cell membrane[195,196]. For example, $G\alpha_{q/11}$ proteins have been reported to increase constitutive activity of the histamine H_1 receptor[197] and have a role in dimerization[198]. Data in Figure 4 clearly indicate that $G\alpha_{q/11}$ proteins, which have been estimated to have a molecular weight of 42 kDa[199], were present in both non-immobilized and immobilized vesicles containing HA-hH_1R-HIS. We can not be certain about the proportion of immobilized $G\alpha_{q/11}$ proteins which were effectively stripped off the resin, because we may only be collecting proteins which were immobilized via other proteins in the membrane, and not those which were directly covalently linked to the resin. However, while not a complete inventory of all of the proteins required for GCPR signalling, the presence of the membrane associated $G\alpha_{q/11}$ proteins is strongly suggestive that other such proteins are likely co-immobilized as well. The presence of other members of the signalling cascade may play a role in maintaining a similar pharmacology for immobilized and non-immobilized receptors (see below) and likely contributes to the enhanced stability of receptors in immobilized vesicle preparations.

The efficiency of immobilization is an important aspect. We readily determined conditions under which the actual cross-linking process is quite efficient. Under these conditions the density

of receptor on the beads is then a simple function of the expression level. However, achieving a high level of functionality of the immobilized receptors required investigation of a number of parameters. Increasing the linker to 8 atoms from 5 yielded a near doubling of the amount of functional receptor immobilized. The reason for the sensitivity to linker length is not clear at this point, however, it is known that GPCRs require a fluid membrane environment[112] and space between the surface and the membrane for extracellular domain movement[143] for full functionality. It therefore seems possible that a short spacer leads to vesicle restriction and alters dynamics and mechanical properties.

When all of the optimisation steps were combined, it proved possible to functionally immobilize these two GPCRs with an efficiency of approximately 25 % in comparison to similarly treated but non-immobilized vesicle preparations. The yield of functional immobilized GPCRs was very similar to what we have observed for model bacterial membrane proteins that have been purified and solubilized in micelles (Chapters 4 - 6). The current yield of 25 % is likely not an upper limit as can be seen from improved functionality upon increasing linker lengths, and further investigation into various matrices and linking chemistries should prove useful for further improvement of the efficiency. Furthermore, although we use a pH that favours the reaction at the N-terminus, the current chemistry may still be affecting some lysines which may be important for activity, such as Lys5.39 in the hH_1 receptor ligand binding site, established from mutagenesis studies[200,201]. The N-terminus itself may also be too close to the binding site and steric hindrance may be limiting the activity of the immobilized GPCRs. However, the major limiting factor at this point appears to be the density at which the receptors were expressed. This issue could best be addressed by investigating other systems geared towards higher level expression. We have focussed on stably transfected mammalian cell culture as a source of material to develop the immobilization protocol. Clearly, one could produce similar vesicles from insect cells expressing the protein of interest at 10 - 100 fold higher levels. We are also investigating the possibility of on-bead enrichment strategies to improve the density of immobilized receptors to a level which would be sufficient for screening GPCRs with TINS.

Immobilization of vesicle bound receptors had had only limited impact on their pharmacology. The immobilized HA-hH_1R-HIS and hA_1R populations bound antagonists and inverse agonists,

with affinity similar to non-immobilized receptors. Interestingly, the proportions of high and low affinity agonist populations typical of non-immobilized receptors[191] seemed to change slightly upon immobilization of the hA_1R while that of the HA-hH_1R-HIS is indistinguishable within the limits of experimental error. At present the basis for this difference is not known but may be related to the co-immobilization of the appropriate G proteins.

Screening of collections of compounds for target binding is often the first step in new drug discovery projects. Presently cell-based assays are the primary method of carrying out such programs for membrane bound targets. A generally applicable procedure for functional immobilization of membrane proteins could potentially be applied in any of the current bead based assays, for example our own NMR-based method[77] (Chapters 4 - 6) or Scintillation Proximity Assays. Use of an appropriate reference such as a parental cell line would enable these methods to be applied to membrane proteins. In some cases a secondary immobilization of the sepharose bead itself may also be necessary. Additional biophysical techniques to detect or characterise ligands for which the present immobilization method could be useful include frontal affinity chromatography-mass spectroscopy[202] and affinity capillary electrophoresis[203]. In addition to ligand screening, functional immobilization could be a powerful tool for deorphanization studies. The method has the potential of being generally applicable to all GPCRs without being G protein or secondary messenger system specific. Although challenging subjects for biophysical applications due to their fragile and exigent nature, GPCRs have, on the contrary, proven to be easy targets for this simple immobilization methodology. This fact is accentuated by the absence of a requirement for tags, biotinylation, or the use of antibodies as tools for immobilization. Furthermore, the increased stability of the receptors upon immobilization will allow longer high throughput screening experiments to be carried out. Our immobilization strategy therefore should enable the study of a broader range of membrane proteins, including GPCRs, in their native membranes using different analytical methods.