# Characterization of radioligand binding to a transmembrane receptor reconstituted into Lipobeads

Paul S.-H. Park[a,1], Charlene C. Ng[a,1], Sean Buck[a], James W. Wells[a], Yu-Ling Cheng[b], Peter S. Pennefather[a,*]

[a] *Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ont., Canada M5S 2S2*
[b] *Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ont., Canada M5S 3E5*

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**Abstract Lipobeads are hydrogel beads surrounded by a lipid bilayer membrane and have been developed to act as a cell analogue. The FLAG-tagged $M_2$ muscarinic receptor was incorporated onto the surface of the Lipobead by incubating pre-Lipobeads with proteoliposomes containing the receptor. Receptors reconstituted onto the surface of the Lipobeads were functional in that they bound the antagonists quinuclidinylbenzilate and scopolamine with characteristic muscarinic affinities. This demonstrates the feasibility of using Lipobeads to study the binding properties of the $M_2$ muscarinic receptor and offers a promising approach to the study of transmembrane protein biology in general.**



*Keywords:* Assay system; Biomaterial; Biosensors and nanotechnology; Muscarinic receptor; Lipobead; Transmembrane receptor

## 1. Introduction

Transmembrane proteins, such as receptors, have a hydrophobic transmembrane region that requires a complementary amphiphilic environment to promote and maintain proper folding and function of the protein. Transmembrane proteins therefore are often studied in preparations of protein-enriched membrane fragments and, upon solubilization, after subsequent reconstitution in liposomes [1,2]. To overcome the mechanical instability associated with these classical methods, and to facilitate ease of handling, supported phospholipid membranes on modified solid surfaces have been developed [3–6]. The solid surfaces used to support phospholipid membranes typically are made up of a flat sheet of glass, silica, or a surface coated with gold. We have reported previously on a method for creating a supported phospholipid membrane on the surface of a microscopic phospholipid hydrogel conjugate [7], which we have called pre-Lipobeads. These are free hydrogel beads formed with a certain density of phospholipids covalently attached to the hydrogel surface. The hydrophobic anchors on the surface of the hydrogel pre-Lipobeads promote the self-assembly of a membrane on the bead surface when exposed to liposomes, resulting in the formation of Lipobeads [7] (Fig. 1A). More recently, we have confirmed that the Lipobead membrane is composed mostly of two leaflets of phospholipid and has the ability to encapsulate large hydrophilic solutes [8]. In the present study, we were interested in determining whether transmembrane receptors could be incorporated onto the surface of these Lipobeads and retain native function.

## 2. Materials and methods

### 2.1. Materials

Anti-FLAG m2-FITC conjugated antibody, carbachol, *N*-methylscopolamine bromide, sodium deoxycholate, sodium cholate, egg phosphatidylcholine (ePC), bovine serum phosphatidylserine (PS), and cholesterol were obtained from Sigma–Aldrich, Canada. Scopolamine hydrobromide and (−)-quinuclidinylbenzilate (QNB) were from RBI-Sigma. [$^3$H]QNB (lot 3363717, 37 Ci/mmol; lot 3467373, 39 Ci/mmol) was obtained from Perkin–Elmer Life Sciences. The fluorescent phospholipid 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine (PC-NBD) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals used were of reagent grade.

### 2.2. Reconstitution of $M_2$ muscarinic receptor in phospholipid vesicles

FLAG- and c-Myc-tagged $M_2$ muscarinic receptors were expressed in *Sf*9 cells and extracted in digitonin-cholate as described previously [9]. Reconstitution was carried out essentially as described by Haga et al. [10]. The following conditions are for reconstitution on a 2-mL bed volume of Sephadex G-50 (fine); they were scaled up accordingly for larger volumes of reconstitution. Sephadex G-50 (fine) was packed in Disposaflex (0.8 cm diameter, Kontes), Poly-Prep (10 mL, Bio-Rad), or Econo-Pac (20 mL, Bio-Rad) columns. Solubilized extracts were concentrated 4–14-fold on Centricon-10 (Millipore) concentrators. The concentrated extract (106–366 nM, 100 μL) was incubated with carbachol (10 mM) for 15 min on ice. Lipid vesicles consisted of 1.26 mg/mL ePC:PS:cholesterol (10:10:1 by wt) in HEN buffer (160 mM NaCl, 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethane-sulfonic acid] (HEPES), and 1 mM ethylenediaminetetraacetic acid (EDTA), pH adjusted to 8.0 with KOH), supplemented with 0.18% sodium deoxycholate and 0.04% sodium cholate, and were prepared by sonication. The receptor and lipid suspension were mixed in a 1:1 ratio (200 μL) and passed down to a column of Sephadex G-50 (fine) (2 mL bed volume) pre-equilibrated with HEN buffer. The column was washed with HEN buffer (600 μL). The reconstituted receptor was collected in the void volume by eluting with an additional 500 μL of HEN buffer. The concentration of reconstituted receptor ranged from 9 to 53 nM, as determined by [$^3$H]QNB.

Fluorescently labeled lipid vesicles (ePC:PS:cholesterol:PC-NBD 11:12:1.2:1 by wt) were prepared in a manner similar to that described

* Corresponding author. Fax: +1-416-978-8511.
*E-mail address:* p.pennefather@utoronto.ca (P.S. Pennefather).

[1] These authors contributed equally to this work.

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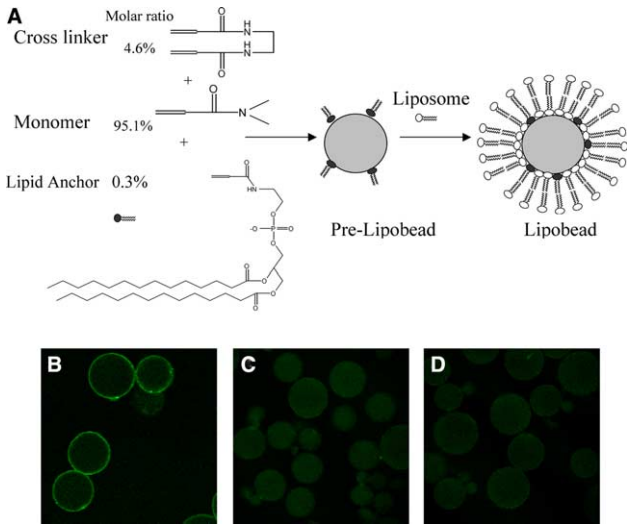


Fig. 1. Reconstitution of FLAG-tagged $M_2$ muscarinic receptor onto the surface of Lipobeads. (A) Schematic illustrating the formation of a Lipobead. Empty phospholipid vesicles (C) or vesicles containing either FLAG-tagged $M_2$ receptor (B) or c-Myc-tagged $M_2$ receptor (D) were reconstituted onto the surface of Lipobeads. The beads were stained with an anti-FLAG antibody conjugated to FITC and viewed on a confocal microscope at a wavelength of 498 nm.

above and passed down to a column of Sephadex G-50 (fine) pre-equilibrated with HEN buffer. The UV absorbance of the last fraction eluted from the column was measured at 464 nm. The phospholipid concentration in this liposome preparation, estimated by UV absorbance spectroscopy, was 0.15 mg/mL or 0.23 mM, assuming an average molecular weight of 650 for the lipid.

### 2.3. Reconstitution of $M_2$ muscarinic receptor on Lipobeads

Pre-Lipobeads were prepared using 5 mol% cross-linked poly(dimethylacrylamide) beads with 33 wt% anchors in the surfactant mixture (total mass of 90 mg) as described by Ng et al. [7]. The dry beads (7 mg) were hydrated with HEN buffer (50 μL) for 10 min. The wetted pre-Lipobeads were then incubated with 300 μL of receptor proteoliposomes for 2 h at room temperature. Unbound vesicles were removed by washing the Lipobeads four times with HEN buffer.

### 2.4. Antibody labeling of the proteolipobeads

The anti-FLAG antibody conjugated to FITC (28 μg/mL) was added to the proteolipobead sample and incubated overnight at 4 °C. The Lipobead membranes remained impermeant to antibody which was directed at an N terminal (extracellular) domain. The samples were then washed four times with HEN buffer prior to confocal microscopy. Laser scanning confocal microscopy images were obtained using a Model 5.10 Carl Zeiss Axiovert 100M laser scanning confocal microscope equipped with a 10×/0.5 NA Fluar lens, an argon laser using the 488 nm line, a beam splitter HFT 488, and an emission filter BP505-530. A pinhole of 120 μm in size was used with the 10× lens, which corresponded to an optical section of 10 μm in thickness.

### 2.5. Binding assays and analysis of data

To obtain proteolipobeads for use in binding assays, proteoliposomes were incubated with carbachol (10 mM) for 15 min on ice prior to their incorporation into the beads. The proteolipobeads were then washed four times with ice-cold HEN buffer (1 mL) to remove free proteoliposomes and carbachol. Solutions of [$^3$H]QNB and any unlabeled ligands (500 μL) were prepared in buffer A (250 mM $KH_2PO_4$, 8 mM EDTA, 100 mM $MgCl_2$, 230 mM NaCl, 4 mM HEPES, and 1 mM phenylmethylsulfonyl fluoride, pH adjusted to 7.60 with KOH) and added to the washed proteolipobeads. The reaction mixture was incubated at 30 °C for 2 h. The reaction was terminated by decanting the supernatant and washing the beads three times with ice-cold buffer A (1 mL). Ready Protein+ (Beckman) scintillant was added to the washed beads (5 mL), which were then assayed for radioactivity. Each sample was counted twice for 10 min by liquid scintillation spectrometry. Assays were performed in duplicate.

Binding to proteoliposomes was measured essentially as described previously [11]. Aliquots of the liposomal preparation (3 μL) were added to buffer A (50 μL) containing [$^3$H]QNB and any unlabeled ligands, and the mixture was incubated at 30 °C for 2 h. Routine estimates of capacity were performed at a saturating concentration of [$^3$H]QNB (~100 nM). Non-specific binding was taken as total binding in the presence of 1 mM unlabeled *N*-methylscopolamine. Bound radioligand was separated on a column of Sephadex G-50 (fine) ($0.8 \times 6.5$ cm) pre-equilibrated and eluted with buffer B (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM $MgSO_4$, and 0.017% digitonin, adjusted to pH 7.40 with NaOH). All of the eluant, including the void volume (1.65 mL), was collected and assayed for radioactivity.

All data were analyzed with total binding taken as the dependent variable ($B_{obsd}$) and with the total concentrations of all ligands taken as the independent variables. Analyses were based on Scheme 1, where the radioligand (P) and an unlabeled ligand (A) compete for distinct and mutually independent sites ($R_j$, $j = 1, 2, \ldots, n$). Sites of type $j$ bind P and A with equilibrium dissociation constants $K_{Pj}$ and $K_{Aj}$, respectively, and constitute the fraction $F_j$ of all sites ($F_j = [R_j]_t/[R]_t$, where $[R]_t = \sum_{j=1}^{n}[R_j]_t$). Total binding was calculated according to Eq. (1), where $B_{sp}$ represents the level of specific binding at the corresponding value of $[P]_t$ and NS is the fraction of unbound radioligand that appears as non-specific binding. Total specific binding was calculated according to Eq. (2) and the required values of $[PR_j]$ were obtained from the equation $[PR_j] = [P][R_j]/K_{Pj}$; the free concentrations of receptor and each ligand ([P], [A]) were calculated numerically from the corresponding total concentrations ($[R]_t$, $[P]_t$, $[A]_t$) [12],

$$B_{obsd} = B_{sp} + NS([P]_t - B_{sp}), \quad (1)$$

$$B_{sp} = \sum_{j=1}^{n}[PR_j]. \quad (2)$$

Further details regarding the analyses and related statistical procedures have been described elsewhere [11–14].

## 3. Results and discussion

It has been shown previously that c-Myc- and FLAG-tagged forms of the human $M_2$ muscarinic receptor can be expressed in *Sf*9 cells and used as a rich source of receptor for biochemical study [9]. The tagged receptor can be solubilized in detergent and retains function in that it binds muscarinic antagonists with characteristic affinities [9,15]. In the current investigation, we have extracted the muscarinic receptor from *Sf*9 cells using digitonin-cholate and have reconstituted the protein into liposomes. When the proteoliposomes are incubated with hydrated pre-Lipobeads, the receptor is incorporated into the membrane that encapsulates the resulting Lipobeads (Fig. 1A). FLAG-tagged receptor was detected at the surface with an anti-FLAG antibody (Fig. 1B). Since the FLAG epitope is at the amino terminus of the protein, at least some receptors detected on the surface of the Lipobead are in a proper orientation, with the amino terminal facing away from the bead core. The anti-FLAG antibody was specific for its epitope, as no staining was detected at the surface of Lipobeads prepared with liposomes lacking receptor or with those containing the c-Myc-tagged $M_2$ muscarinic receptor (Fig. 1C and D).

$$PR_j \underset{[P]/K_{Pj}}{\rightleftharpoons} R_j \overset{[A]/K_{Aj}}{\rightleftharpoons} AR_j$$

Scheme 1.

Receptors in liposomal vesicles and Lipobeads were characterized in binding assays with the antagonists QNB and scopolamine. The equilibrium dissociation constant of each ligand was inferred from its inhibitory effect on the specific binding of [$^3$H]QNB (Fig. 2). In both formulations, the measured muscarinic affinities were similar to those observed previously with extracts of muscarinic receptor from porcine atria (Table 1) [11]. The efficiency of reconstitution of receptors into liposomes was 35–98% with a mean of $61 \pm 8\%$ ($N = 7$), as determined by [$^3$H]QNB. The efficiency for incorporation of the reconstituted receptor into Lipobeads was 0.033–0.16% (Table 2).

The total concentration of reconstituted receptor in liposomal preparations was 8–50 nM and the corresponding lipid concentration was estimated to be 0.23 mM. Thus, there was one receptor for every 4000–25 000 molecules of lipid. The radius of a liposome was 15 nm as determined by dynamic light scattering and the surface area was therefore $2.8 \times 10^3$ nm$^2$. Since the surface area occupied by a single lipid molecule in a phospholipid bilayer is roughly 0.60 nm$^2$ [16], each liposome contained about 4700 lipid molecules per leaflet of bilayer or 9400 lipid molecules overall. Each 300 μL aliquot of the preparation therefore contained about $4.2 \times 10^{16}$ molecules of lipid, $4.5 \times 10^{12}$ liposomes, and from $1.5 \times 10^{12}$ to $8.9 \times 10^{12}$ molecules of receptor. It follows that there were 0.3–2 receptors per liposome.

Table 2
Incorporation of receptor into liposomes and Lipobeads

| Preparation | FLAG-$M_2$ muscarinic receptor | | Efficiency[a] (%) |
|---|---|---|---|
| | Proteoliposome (pmol) | Proteolipobead (fmol) | |
| A | 2.5 | 3.0 | 0.12 |
| B | 2.9 | 4.4 | 0.15 |
| C | 4.6 | 7.4 | 0.16 |
| D | 5.5 | 6.0 | 0.11 |
| E | 7.0 | 4.6 | 0.066 |
| F | 14.2 | 4.6 | 0.033 |

An aliquot of the proteoliposomal preparation (300 μL) was mixed with pre-Lipobeads (7 mg, dry weight) as described in Section 2. The amount of receptor taken from the liposomal preparation was inferred from the best fit of Scheme 1 to the data represented in Fig. 2A (i.e., $[R]_t$) or estimated from the specific binding at a near-saturating concentration of [$^3$H]QNB (~100 nM). The amount of receptor incorporated into Lipobeads was inferred from the best fit of Scheme 1 to the data represented in Fig. 2B (i.e., $[R_1]_t$). The mean value for the six preparations of proteolipobead is $5.0 \pm 0.6$ fmol in a volume of 300 μL.
[a] Efficiency of transfer of receptor from liposomes to Lipobeads.

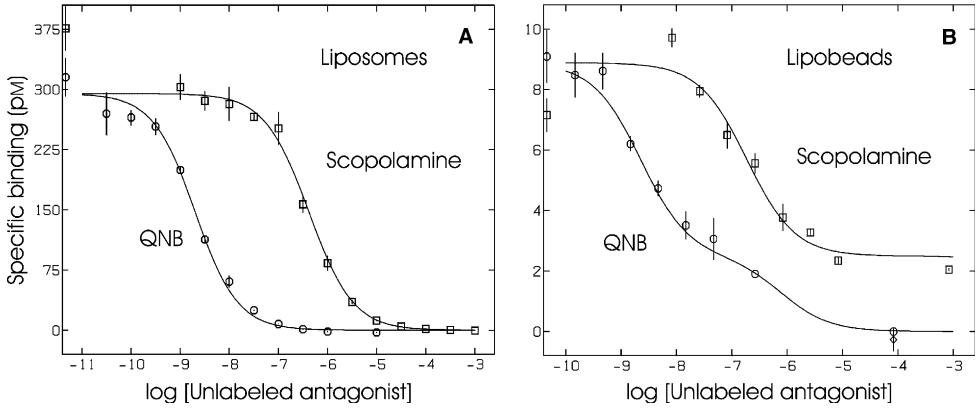


Fig. 2. Binding of QNB and scopolamine to FLAG-tagged $M_2$ muscarinic receptor. Membranes from *Sf*9 cells expressing the receptor were solubilized in digitonin-cholate and reconstituted in phospholipid vesicles (A) and Lipobeads (B). Total binding was measured at a sub-saturating concentration of [$^3$H]QNB and graded concentrations of either unlabeled QNB (○) or scopolamine (□). A single point at the concentration of unlabeled QNB shown in (B) (◇) was measured in parallel with each full curve measured in the presence of scopolamine. The data for both ligands in each panel were analyzed simultaneously and the lines represent the best fits of Eq. (1). Single values of $K_{Lj}$ were common to all of the data acquired with the same ligand (L ≡ P or A) and it was assumed that $K_P = K_A$ for QNB. A single value of $F_j$ was common to all of the data and a separate value of $[R]_t$ was assigned to the data from each experiment. Each point represents the mean of either three or four experiments (±S.E.M.). Individual estimates of $B_{obsd}$ were adjusted as described previously to obtain the corresponding values of $B_{sp}$ plotted on the *y*-axis [10]. The value of $[R]_t$ used in the adjustment was taken as the mean of the individual values from the experiments represented in each of the panels (A, $352 \pm 45$ pM, $N = 7$; B, $1.2 \pm 0.1$ nM, $N = 6$). The mean values of $\log[P]_t$ used for the adjustments in (A) and (B) are $-8.78 \pm 0.03$ and $-8.76 \pm 0.04$, respectively. Points at the lower end of the *x*-axis indicate binding in the absence of unlabeled ligand. The parametric values are listed in Table 1.

Table 1
Affinities of QNB and scopolamine for FLAG-tagged $M_2$ muscarinic receptor

| Preparation | QNB | | Scopolamine | | |
|---|---|---|---|---|---|
| | $\log K_{P1}$ | $\log K_{P2}$ | $\log K_{A1}$ | $\log K_{A2}$ | $F_1$ |
| Proteoliposome | $-9.58 \pm 0.20$ | –[a] | $-7.22 \pm 0.19$ | –[a] | –[a] |
| Proteolipobead | $-9.28 \pm 0.70$ | $-6.10 \pm 0.71$ | $-7.36 \pm 0.61$ | –[b] | $0.007 \pm 0.01$[c] |

The parametric values from the analyses represented in Fig. 2 are listed in the table.
[a] One class of sites was sufficient for Scheme 1 to describe the data.
[b] The value of log $K_{A2}$ was undefined and was fixed accordingly during the fitting procedure (i.e., $\log K_{A2} > -1$).
[c] Fraction of sites exhibiting higher affinity for QNB (i.e., $K_{P1}$) and corresponding to the $M_2$ muscarinic receptor. The mean values of $[R_1]$ and $[R_2]_t$ are $6.7 \pm 0.9$ pM and $0.92 \pm 0.12$ nM ($N = 3$), respectively.

Specific binding of [$^3$H]QNB to receptor-containing Lipobeads could be detected only when the proteoliposomes were pre-incubated with the muscarinic agonist carbachol (10 mM) prior to reconstitution with the beads. No specific binding was observed when the Lipobeads were reconstituted with proteoliposomes that either were not pre-incubated with ligand or were pre-incubated with the antagonist *N*-methylscopolamine (1 μM) or [$^3$H]QNB (3 nM). This suggests that the incorporation of functional receptor onto the surface of Lipobeads requires a specific conformation that is favored by agonists but not antagonists.

Binding of QNB to Lipobeads reconstituted with FLAG-tagged receptor revealed two populations of sites. The sites of higher affinity resembled the receptor in phospholipid vesicles (i.e., $K_{P1} = 0.3$–$0.5$ nM, Table 1), whereas those of weaker affinity were found only with the beads ($K_{P2} = 0.8$ μM). The latter accounted for 28% of saturable binding at the concentration of [$^3$H]QNB used in the assays (Fig. 2B), or 99.3% of the total capacity for the radioligand, and they presumably derive from the bead itself. Binding of QNB to hydrated pre-Lipobeads not exposed to liposomes revealed an affinity that was comparable to that of the low-affinity site detected in the proteolipobeads (i.e., 0.1 μM); also, the level of binding increased with the level of cross-linking in the polymer (not shown). This suggests that the cross-linked polymer forms hydrophobic pockets that can bind QNB with a weak affinity.

In contrast to QNB, the antagonist scopolamine bound to a uniform population of sites on the reconstituted Lipobeads (Fig. 2B). The affinity of scopolamine for receptor on the beads was the same as that for receptor in liposomes (Table 1) and the corresponding capacity was equal to that of the high-affinity sites revealed by QNB (Fig. 2B). Scopolamine is more hydrophilic than QNB and therefore would be expected to have a weaker affinity for non-receptor binding sites within the core.

Receptors reconstituted onto the surface of the Lipobeads therefore appear to be functional in that they bind muscarinic antagonists with characteristic affinities. The binding pocket of the receptor is thought to be located approximately 9 Å beneath the membrane surface [17] and its functional viability very likely requires the proper arrangement of the seven transmembrane α-helices. The retention of binding suggests that neither the reconstitution procedure nor the presence of the underlying hydrogel support caused significant distortion of protein folding, at least when the receptor was protected with carbachol during reconstitution.

A minority of receptors was transferred from liposomes to Lipobeads (Table 2) and the low efficiency can be attributed in part to a ceiling imposed by the surface area of the latter. As described above, a single liposome has a surface area of $2.8 \times 10^{-3}$ μm$^2$ (radius 0.015 μm). Since the Lipobeads used here have a surface area of approximately $3.1 \times 10^4$ μm$^2$ (radius 50 μm), about $1.1 \times 10^7$ liposomes will be required to generate a bilayer over the entire surface of a Lipobead. If a 300 μL aliquot contains $4.5 \times 10^{12}$ liposomes (see above), complete uptake of the liposomes onto Lipobeads will require about $4.1 \times 10^5$ beads. The actual number of Lipobeads can be estimated from the total volume after hydration and the volume per bead. In hydrated Lipobeads, the measured volume fraction of polymer was 20% (vol. polymer/vol. bead). We therefore expect that 7 mg of dry beads will yield 0.035 cm$^3$ (35 μL) of hydrated beads, given that Lipobeads have a density of 1 g/cm$^3$. Since the volume of a single bead is $5.2 \times 10^{-7}$ cm$^3$, based on a radius of 0.005 cm, the mixture must contain about $6.7 \times 10^4$ beads, or 6-fold fewer than required to accommodate all available liposomes. This suggests that a smaller amount of proteoliposome could have been used to transfer the same number of receptors to the Lipobeads, although a longer incubation time might be required for the liposomes to find and to fuse with the beads.

The observed incorporation of receptor into Lipobeads was 5 pmol (Table 2, or $0.045 \times 10^6$ receptors per bead based on a total of 67 000 beads. The predicted density is $3.6 \times 10^6$ receptors per bead at the lowest concentration of receptor (i.e., 8 nM or $1.5 \times 10^{12}$ receptors in 300 μL), assuming that the level of incorporation is limited by the number of beads. The predicted density is $23 \times 10^6$ receptors per bead at the same concentration of receptor, assuming that there are sufficient beads to accommodate all of the proteoliposomes. The bead-limited density is therefore less than the theoretical maximum for that quantity of proteoliposomes, but it remains 80-fold greater than the measured density. The source of this latter discrepancy is unclear. It may derive from the inactivation of some receptors upon their incorporation into the Lipobeads or from a failure of some receptors to accompany the liposome lipid into the Lipobead membrane. In any event, the data suggest that there is potential for a substantial increase in the ratio of signal to noise.

The G protein-coupled receptors (GPCRs) are a major target for drug discovery [18]. A number of molecular assays, in which purified GPCRs are reconstituted into a membrane preparation, have been reported previously. Corning has a receptor array in which GPCRs are incorporated into a supported lipid bilayer [19]. Biacore has produced a variant of their surface plasmon resonance (SPR) technology where a dextran coating on their SPR electrode is modified with surface lipids that promote the fusion of solubilized GPCRs [20]. Nimbus Inc. reconstitutes transmembrane proteins into lipid bilayers supported on porous silica cores [21], while Biovectors Therapeutics S.A. uses polysaccharide cores [22]. Our method represents an alternative approach to developing binding assays in which GPCRs are reconstituted into an easily manipulated lipid bilayer environment.

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