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# HDL-like discs for assaying membrane proteins in drug discovery

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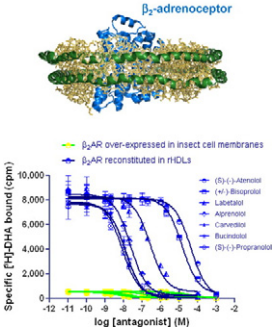
## HIGHLIGHTS

- A SPA-based homogeneous assay allowing the study of ligand binding to the $\beta_2$AR.
- $\beta_2$AR has the same pharmacology in rHDLs than in cell membranes for a set of antagonists.
- Antagonist binding is right-shifted to lower affinities for the solubilized receptor.
- rHDL-like discs can be used for compound screening and lead optimization.

## GRAPHICAL ABSTRACT

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## ABSTRACT

To broaden the use of the recombinant high-density lipoprotein (rHDL) approach to the characterization of lead compounds, we investigated the pharmacology of the human beta-2-adrenoceptor in nanolipid bilayers (rHDL) with a broad set of beta-adrenoceptor antagonists. To that end, we developed a homogeneous copper-chelate scintillation proximity binding assay (SPA) in order to compare receptor-ligand binding affinities before and after reconstitution into rHDLs. Our results clearly show that the beta-2-adrenoceptor reconstituted in rHDLs display the same pharmacology as that in cell membranes and that rHDLs can be used not only to measure affinities for a range of ligands but also to study binding kinetics.



## 1. Introduction

Membrane-spanning proteins are important drug targets, as their activities and the pathways they mediate are relevant in regulating disease processes. There are technical limitations in the mechanistic understanding and application of membrane proteins to drug discovery because of the difficulties in isolating them from the membrane while maintaining function. Thus, drug discovery on membrane proteins is mainly carried out using cells expressing the membrane protein of interest or membrane preparations carrying a mixture of various endogenously expressed proteins. Therefore, the execution and interpretation of biochemical assays on membrane proteins is hampered by the presence of endogenous, related proteins and indirect effects. Furthermore, thermodynamic and kinetic binding studies are powerful tools in drug discovery but interpretation is complex and further complicated by the use of recombinant membrane preparations or tissue which may contain related receptor subtypes.

To study the function of isolated membrane proteins, a new technology has emerged: the so-called membrane discs or reconstituted

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high density lipoprotein (rHDL) particles [1–10]. In this approach, purified membrane proteins are assembled into a nanoscale phospholipid bilayer which is highly soluble in aqueous solution. These rHDLs are composed of approximately 160 phospholipids organized in a bilayer, which is encircled by two apolipoprotein A-I molecules (Fig. 1). The technology has been mainly applied to understand the function of single receptors [6–9] versus dimers [11] or other oligomeric species [12]. However, all these ligand-receptor studies were each limited to a few compounds only. An application to drug discovery has been hampered by the difficulty of preparation of rHDLs and the lack of suitable, higher throughput assays. The human $\beta_2$-adrenoceptor ($\beta_2$AR) is a frequently used model system in these studies [6,9]. $\beta_2$AR is an integral membrane, G-protein coupled receptor (GPCR), that is the focus of intense efforts in the field of asthma and COPD drug discovery and tools that allow detailed studies of receptor-ligand interaction are thus of great interest.

Our ultimate objective is to broaden the rHDL-approach to the characterization of lead compounds in order to facilitate membrane protein drug discovery. Here, we describe a strategy that overcomes these obstacles by combining optimized receptor expression, purification and reconstitution protocols with a commonly used assay technology (SPA). The resulting, novel, assay format is highly sensitive and high throughput-compatible. It not only allowed to measure binding affinities for a range of ligands of the $\beta_2$AR, but also to study binding kinetics.

Following their extraction from the membrane, receptors cannot be studied by classical filtration techniques as solubilized receptors in a detergent micelle or in a rHDL are about 10 nm in diameter and pass through the filter pores. As a consequence, the determination of receptor function in detergents or in nanobilayers has been limited to laborious equilibrium dialysis or gel-filtration methods. Here, we describe the development of a copper-chelate affinity-based scintillation proximity assay (SPA) to study ligand binding to the $\beta_2$AR in the membrane-like environment of a rHDL. This homogeneous radiotracer-binding assay was suitable for quick measurements and further used to optimize the reconstitution conditions of the purified receptor in rHDLs. In SPA, scintillant is incorporated into small fluomicrospheres or beads. If a radioactive molecule is bound to the bead, it is brought into close enough proximity that it can stimulate the scintillant contained within to emit light. Otherwise, the unbound radioactivity is too distant, the energy released is dissipated before reaching the bead, and these disintegrations are not detected. SPA binding assays avoid the usual filtration or washing procedures and are therefore often used in drug discovery where high throughput and ease of automation are required.

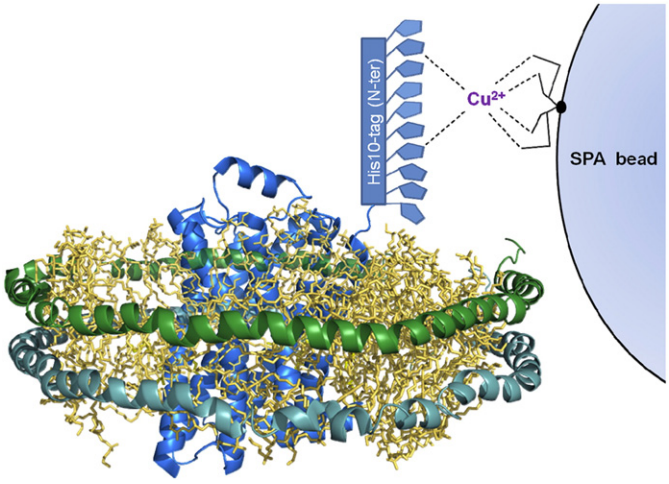


**Fig. 1.** Molecular model of the $\beta_2$AR reconstituted into a recombinant HDL particle (Protein Data Bank ID: 3D4S, 3J00). The receptor is rendered in blue color and the phospholipids are shown in orange. rHDLs are composed of a dimer of an amphipathic scaffold protein, called apolipoprotein (apoA-I), wrapped around a phospholipid bilayer composed of ~160 phospholipid molecules. Each apoA-I protein (cyan and green) is depicted as a ribbon diagram. Apolipoproteins have been shown to associate with phospholipids in vitro, self-assembling in discoidal-shaped particles very similar to the HDL vesicles produced by the liver, which enable lipids like cholesterol and triglycerides to be transported within the bloodstream. Recombinant HDLs can incorporate single molecules of integral membrane protein targets like the $\beta_2$AR through chemical self-assembly (see Methods). The resultant nanostructures, with diameters ranging from 10 to 20 nm, represent a highly stable and homogeneous population with an aqueous solubility in the millimolar range. Copper-chelate SPA beads (2–5 μM in diameter) are used to trap and quantify the association of radiolabelled ligands to the $His_{10}$-tagged $\beta_2$-adrenoceptor. Figures were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.).

## 2. Materials and methods

### 2.1. Cloning of human apoA-I and $\beta_2$AR

A codon optimized version of human apolipoprotein A-I (Uniprot: P02647) was cloned into a pET41a (Merck) derived *E. coli* expression vector using Gateway recombination technology [13]. A DNA fragment encoding APOA1 amino acids L68-Q267 was made by PCR with the primer pairs fwd-gatccCTGAAGCTGTTGGACAAT/rev-gcATTACTGGGTATTCAGCTT and fwd-cCTGAAGCTGTTGGACAAT/rev-ggccgcATTACTGGGTATTCAGCTT, respectively. The two PCR products were gel purified (NucleoSpin Extract II, Macherey-Nagel), unified, denatured and reannealed [14]. The resulting sticky-end PCR product was subsequently ligated with the large *Bam*HI-*Not*I fragment of the entry vector pGENT-PE. In a Gateway LR reaction, the gene of interest was subcloned into the expression vector pHisKan5. DNA encoding human $\beta_2$-adrenoceptor (RefSeq: NM_000024) was subcloned into a mammalian expression vector and into a baculovirus donor vector. Both vectors were previously prepared. The mammalian vector pACA-M11 was derived from pCMVSport1 (Invitrogen) whereas the baculo vector pACA-BG11 was derived from pBacPAK8 (Clontech) and features EGFP co-expression [15]. Both plasmids have a cleavable N-terminal Flag-$His_{10}$ tag with a hemagglutinin signal peptide for protein secretion. The target gene was amplified by PCR using the primer pairs fwd-gggcCAACCAGGTAACGGCAG/rev-ttaCAGCAGTGAGTCATTTGTACTA and fwd-CAACCAGGTAACGGCAG/rev-aaccttaCAGCAGTGAGTCATTTGTACTA, respectively. Cloning was completed as described above via the type IIs restriction sites *Esp*3I of the expression vectors.

### 2.2. Expression and purification of apoA-I

Briefly, 50 ml of LB medium containing 50 μg/ml kanamycin were inoculated with a single colony from a fresh plate and the culture was grown at 37 °C with shaking (200 rpm) until $OD_{600}$ reached 0.6–0.8 after 4–6 h. Two liters of sterilized TB medium, 50 μg/ml kanamycin were then immediately inoculated with 20 ml of starting culture and incubated at 37 °C with shaking. When $OD_{600}$ reached 0.6 (~3 h), the temperature was lowered to 25 °C for 30 min before the culture was induced overnight with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were then harvested by centrifugation at 8000 ×*g* for 20 min and stored at −80 °C. For purification, 15-20 g of cells were resuspended in 200 ml of 50 mM sodium phosphate buffer pH 8.0 containing 300 mM NaCl, 20 mM imidazole, 5000 U benzonase (Merck) and complete protease inhibitor cocktail tablets (Roche). The cells were homogenized for 30 s with a Polytron PT-2100 homogenizer and lysed by two passages through an Avestin cell disruptor (10,000-15,000 psi). 0.5 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (Roche) and 1% Triton X-100 (Fluka) were added (30 min incubation with shaking at 4 °C) and the lysate was cleared by centrifugation at 25,000 ×*g* for 30 min. The cleared lysate was loaded onto 2×5 ml HisTrap HP columns (GE Healthcare). The columns were washed with 10 bed volumes (BV) of 50 mM sodium phosphate, pH