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Detergent-free extraction of a functional low-expressing GPCR from a human cell line

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Abstract

Dopamine receptors (DRs) are class A G-Protein Coupled Receptors (GPCRs) prevalent in the central nervous system (CNS). These receptors mediate physiological functions ranging from voluntary movement and reward recognition to hormonal regulation and hypertension. Drugs targeting dopaminergic neurotransmission have been employed to treat several neurological and psychiatric disorders, including Parkinson's disease, schizophrenia, Huntington's disease, attention deficit hyperactivity disorder (ADHD), and Tourette's syndrome. In vivo, incorporation of GPCRs into lipid membranes is known to be key to their biological function and, by inference, maintenance of their tertiary structure. A further significant challenge in the structural and biochemical characterization of human DRs is their low levels of expression in mammalian cells. Thus, the purification and enrichment of DRs whilst retaining their structural integrity and function is highly desirable for biophysical studies. A promising new approach is the use of styrene–maleic acid (SMA) copolymer to solubilize GPCRs directly in their native environment, to produce polymer-assembled Lipodisqs (LQs). We have developed a novel methodology to yield detergent-free D1-containing Lipodisqs directly from HEK293f cells expressing wild-type human dopamine receptor 1 (D1). We demonstrate that D1 in the Lipodisq retains activity comparable to that in the native environment and report, for the first time, the affinity constant for the interaction of the peptide neurotransmitter neurotensin (NT) with D1, in the native state.

Keywords: GPCR, Dopamine receptor, Lipodisq, Microscale thermophoresis, detergent-free, neurotensin

Highlights

1. We report for the first time the extraction and purification of a wild-type human GPCR from its native lipid environment via detergent-free Lipodisq formation.
2. We demonstrate that microscale thermophoresis can be successfully employed to characterize ligand binding to a Lipodisq-embedded protein.
3. We show for the first time the binding of the neurotransmitter neurotensin to D1, an interaction that might have important biological implications.

1. Introduction

GPCRs are seven transmembrane proteins and constitute the largest class of cell surface receptors, comprising approximately 4% of the human genome[1]. In humans, approximately 400 GPCRs are known to bind endogenous ligands and approximately 500 GPCRs are responsible for either olfaction or taste[2]. The heterogeneity of these GPCRs and the conformations they can adopt ensures the broad recognition of multiple external stimuli such as taste, smell, light, pain or internal stimuli such as hormone secretion or neurotransmission[3,4]. Due to their participation in nearly all cellular signalling events, they are of major interest to the pharmaceutical industry, with approximately 40% of all current marketed drugs acting as modulators of approximately 5% of known GPCRs[5,6].

Dopamine receptors (DRs) which are class A GPCRs, are characterized by an extracellular N-terminus containing several conserved cysteine residues, which stabilize the protein structure, and a very long intracellular C-terminus [7]. The C-terminus is involved in different signalling events in the cell and upon its phosphorylation the receptor is internalized via the β-arrestin pathway[8–10]. These receptors are prevalent in the central nervous system (CNS) and mediate several physiological functions such as voluntary movement, reward, hormonal regulation and hypertension[7,9,11]. Drugs targeting dopaminergic neurotransmission have been employed to treat several neurological and psychiatric disorders, including Huntington's and Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder (ADHD) and Tourette's syndrome[12]. DRs are also targets for studying drug abuse or addiction[9].

DRs are divided into two subfamilies, the D1 family (D1 and D5) and the D2 family (D2, D3, D4). Their assignment to either subfamily is based on their ability to modulate intracellular concentrations of cAMP. They are thought to be present in the membrane as monomers but also homodimers, heterodimers or tetramers, as evidenced by fluorescence and bioluminescence experiments using both *in vivo* and *in vitro* models[7,13–15]. As an example, the cross family hetero-oligomerization of D1 and D2 receptor is thought to play a role in drug addiction phenomena by re-programming the signalling transduction cascades. These receptors can also hetero-oligomerize with other GPCRs such as the opioid, cannabinoid, histamine or neurotensin receptors, making their individual study problematic due to their promiscuous interactions and potential for involvement in multiple signalling pathways[16–18].

Although DRs have been extensively studied for nearly 40 years (discovery of the first dopamine receptor was in 1975[19]), only a few crystal structures have been published to date (for D3[20], D4[21] and recently D2[22]) hampering efforts to fully elucidate the modes of ligand binding and therefore the design of more potent drugs or inhibitors.

Indeed, the majority of the studies on DRs have been performed using *in vitro* models such as cell culture or animal tissues, fewer studies have been performed using detergent matrices, due to their poor stability in the detergent environment, evidenced by loss of function. Expression of sufficient quantities of functional receptor for biophysical characterization and crystallization is also a significant obstacle. Several approaches have been developed over recent years in efforts to improve the expression of GPCRs, including the change of the host expression organism from bacterial to mammalian sources[23,24]; introduction of a soluble protein into the host sequence [23,25,26] either by adding a GFP-tag to one of the protein termini or by introducing T4 Lysozyme in one of the interhelix loops of the protein to promote crystal contacts for crystallography and even mutations of the protein sequence in order to obtain thermostabilized derivatives[27]. However, these approaches may have detrimental effects on ligand binding or signalling[28–30].

SMA polymer has recently emerged as a useful new tool for simultaneous and nonselective extraction of lipids and embedded proteins present from biological membranes, whilst preserving protein structure and activity[31–36]. SMA nanoparticles have also been used for structural characterization by cryo-EM[37,38] and X-ray crystallography[39]. Several membrane proteins including ion channels[40] and GPCRs[41,42] have been studied and characterized from different organisms[43–46]. Proteomics and lipidomics experiments using SMALPs have also been performed using hydrogen-deuterium exchange mass spectrometry, MALDI-TOF MS/MS and using LC-MS/MS[47–50].

The potential to apply this approach to retain a non-thermostabilized receptor, expressed in a mammalian cell line, in a lipid composition wrested from its native environment is therefore scientifically very appealing. Here we show that the SMA polymer is a powerful tool to extract and purify membrane proteins expressed in HEK cells. The D1-Lipodisqs are suitable for standard biophysical techniques such as circular dichroism, radio-ligand binding and microscale thermophoresis. In conclusion, our results show that Lipodisqs represent a robust

nanoplatform for challenging receptors such as GPCRs, as they maintain them intact in their native lipid environment which is crucial for retention of activity.

2. Materials and Methods

2.1 Wild-type human D1

Dopamine receptor 1 (sequence can be found in SI) was expressed by UCB Celltech (Braine, Belgium). Briefly, cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Cells were grown in DMEM F12+ GlutaMAX-I medium (GIBCO, Invitrogen, Merelbeke, Belgium) containing 10% fetal bovine serum (FBS) (BioWhittaker, Lonza, Verviers, Belgium), 400 µg/ml Geneticin (GIBCO, Invitrogen, Merelbeke, Belgium), 100 IU/ml Penicillin and 100 IU/ml Streptomycin (Pen-Strep solution, BioWhittaker, Lonza, Verviers, Belgium). HEK293f cells expressing the human dopamine receptor 1 were developed in house. Adherent cells were cultured in 175 cm^2 Petri dishes until confluent, and the medium removed. The cells were washed with 30 mL phosphate buffered saline (PBS) at 25°C and detached by incubation with 30 mL of 1 mM EDTA solution in PBS (pH 7.4) for 7 minutes at 37°C, and centrifuged (1500*g*, 10 min, 4°C).

2.2 Membrane preparation

Cell pellets (from 500 mL of culture) were resuspended in 3 mL of cold homogenization buffer (20 mM PBS pH 7.4, 2 mM EDTA, and 2 µL/mL of aprotinin, pepstatin and leupeptin protease inhibitors), and then dounce homogenized (100 strokes) on ice. The homogenate was centrifuged (1000*g*, 15 min, 4°C), and the resulting supernatant ultracentrifuged (40,000*g*, 1 h, 4°C). The resulting pellet was resuspended in buffer (50 mM NaH_2PO_4, 200 mM NaCl, pH 8 and 2 µL/mL of aprotinin, pepstatin and leupeptin protease inhibitors) using a 25-gauge needle (100 passes). The total concentration of protein was determined by BCA assay. Resuspended membranes were either directly used for Lipodisq formation or flash frozen in liquid nitrogen or stored at -80°C.

2.3 SMA hydrolysis

Styrene-maleic anhydride polymer (SMAnh, kindly provided by Malvern Cosmeceutics) in a ratio of styrene to maleic anhydride of 3:1, was hydrolysed by adding 1M NaOH solution (5% w/v final) (Fisher), heated at 80-90°C for 1-2 h. 5 M HCl was then added to precipitate the SMA, which was pelleted by centrifugation (2000*g*, 5 min, RT). Pellet was re-solubilised in water and subjected to multiple washing (3-5 times) and centrifugation steps (2000*g*, 5 minutes). Once washed, double distilled water (ddH_2O) was added to the solution and dialysis was performed

overnight in order to remove the excess of salt and to adjust the pH. At this stage, the SMA should be a clear yellowish solution and to further concentrate it, lyophilization was performed. A white powder was collected and weighted. ddH_2O water or buffer was added to a final concentration of 125 mg/ml and pH adjusted to pH 8.

2.4 Lipodisqs formation and purification

Lipodisqs were formed by directly adding the copolymer (styrene-maleic acid, SMA) 3:1 pH 8 to the previously prepared membranes, at a membrane:SMA w/w ratio of 1:1.5. Sample was left to incubate (8 h, 4°C) in a rotating wheel in 50 mM NaH_2PO_4, 200 mM NaCl, pH 8. To remove non-solubilized membrane particles and any aggregated material, ultra-centrifugation was performed (100,000*g*, 40 min, 4°C) prior to size exclusion chromatography. The supernatant was subjected to SEC on a Superdex 200 Increase 10/300 GL (GE Healthcare) to remove excess polymer. Fractions from size exclusion chromatography (from 9 mL to 13 mL elution volume) were collected and applied to a 5 mL HisTrap HP (GE Healthcare) column previously equilibrated in 50 mM PBS buffer. Samples were recirculated through the column at 1.5-2 mL/min for 1-2 hours at 4°C. For elution, increasing concentration of imidazole in binding buffer were employed, using 2 column volumes per concentration gradient. Elution peak appeared between 50 to 500 mM imidazole. D1-Lipodisqs were concentrated using a 100k MWCO Vivaspin20 tube (Greiner) and buffer-exchanged to PBS.

2.5 Radioligand binding assays

The determination of the amount of active receptor was carried out by radioligand binding assays on HEK293f D1 membrane samples and D1 contained Lipodisq. The D1 selective antagonist N-methyl-[^{3}H]-SCH 23390 was used as radioligand (81.9 Ci/mmol, PerkinElmer). For saturation studies, 5 different concentrations of radioligand (in duplicates or triplicates) were used within the 0.2-5 nM range, in 50 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM NaCl buffer at pH 7.4 (avoiding $MgCl_2$ for Lipodisq samples). Protein amount used was 5 µg total protein per sample. Non- specific binding was assayed by competition with excess (10 µM) of unlabelled D1 selective antagonist (+)-butaclamol. All samples were incubated for 1 hour at room temperature for maximum receptor saturation. For the membrane assays, the bound ligand was separated from the free radioligand by rapid vacuum filtration through GF/B or GF/C glass microfiber filters presoaked in polyethyleneimine (PEI) 0.3% for 1 hour to reduce non-specific binding, and the filters were washed 4 times

with 2 mL ice-cold 50 mM NaH_2PO_4 buffer pH 7.4. For Lipodisq samples, the separation was carried out using pre-equilibrated P-30 spin columns (Bio-Rad). Scintillation liquid (3 mL) (ScintiSafe 3 Liquid Scintillation Cocktail, Fisher Scientific) was added to the bound sample and radioactivity was measured in dpm on a scintillation counter (1409 DSA Liquid Scintillation Counter, PerkinElmer). Specific binding was determined by subtracting the non-specific dpm from the total dpm.

2.6 Microscale thermophoresis experiment (MST)

Microscale thermophoresis experiments were performed on a blue/red Monolith NT.115 instrument (NanoTemper Technologies). A typical experiment is carried using a dilution series (10-15 points) of the unlabelled binding partner. LoBind tubes were used to ensure minimal adsorption of the sample in the reaction tubes. Capillary scans were performed to ensure that fluorescence was constant (within ± 10%) within the tubes. The fluorescently labelled ligand Cy5-neurotensin (Cy5-NT) was prepared in 20 mM phosphate buffer 200 mM NaCl pH 8 at the desired concentration (from 0.05 nM to 1 µM). Samples were incubated at 20°C for 30 minutes in binding buffer after which they were loaded into Standard Treated capillaries (NanoTemper Technologies). Thermophoresis was measured at different MST power (20, 40, and 80%), with 80% giving the best results. To account for the non-specific signal, two control experiments were accomplished, one consisting in boiling the LQs samples at 100°C for 10 min, and the other measuring the thermophoresis of LQs prepared from non-transfected HEK293 membranes. The MST data was analysed and the K_D was calculated using the NTAnalysis software (NanoTemper Technologies).

3. Results and Discussion

3.1 Preparation, purification and characterisation of D1 Lipodisqs

The solubilization of the membrane protein was performed by the addition of an excess of polymer to the membrane in a 1:1.5 weight-to-weight ratio as described in the literature[31,34]. The time of incubation of the polymer-membrane mixture was chosen to be 1-2 h at 4°C in order to keep the protein fold intact during the extraction. Although, longer incubation times or higher temperatures could be used for solubilizing membrane proteins from the membrane environment, this can be detrimental to protein folding and activity[31,34,51]. In a few literature examples this solubilization step is performed at room temperature for an extended time, such as for chromatophore (1h at 20°C[45]), complex IV (30 minutes at 25°C[46]) and for the A_{2a} model GPCR in yeast and HEK cells (20h at 20°C[41]). In our case, we chose not to follow the same experimental procedure of Jamshad et al.[41] , as, when compared to A_{2a} for which ligand binding data suggests that the protein retains activity above room temperature, there is no data available for D1 concerning its stability at room temperature or above. Therefore, we decided to keep the temperature as low as possible whilst the SMA incubation time was increased up to 8 hours to maximize the formation of the Lipodisqs at low temperature.

It is important to highlight that the buffer used for membrane solubilization by SMA should contain between 100-300 mM NaCl, as salt allows the polymer to deposit on the membrane by shielding the electrostatic repulsion between SMA and negatively charged lipids, and thus initiate Lipodisq formation[32,35,52].

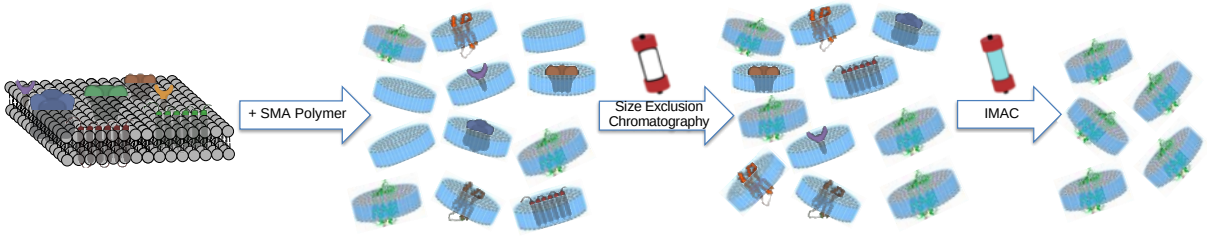

Figure 1 Schematic representation of how to purify membrane proteins using SMA. First, SMA is added to the host membrane, which form proteo-Lipodisq. Excess of SMA is removed via SEC and finally proteo-Lipodisqs are purified via IMAC.

Once the samples were solubilized, any non-solubilized materials and/or aggregates were removed by ultra-centrifugation. The supernatant was subjected to size-exclusion chromatography to remove the excess of free polymer prior to IMAC purification (Figure 1 and 2). Free SMA must be removed for two main reasons: (i) the excess of polymer would unduly complicate the biophysical characterisation of the proteins in Lipodisqs (by CD, for example), and (ii) the maleic acid moiety of SMA would compete with the protein His-tag in binding to the metal ions of the IMAC column, potentially resulting in a poor purification yield[34].

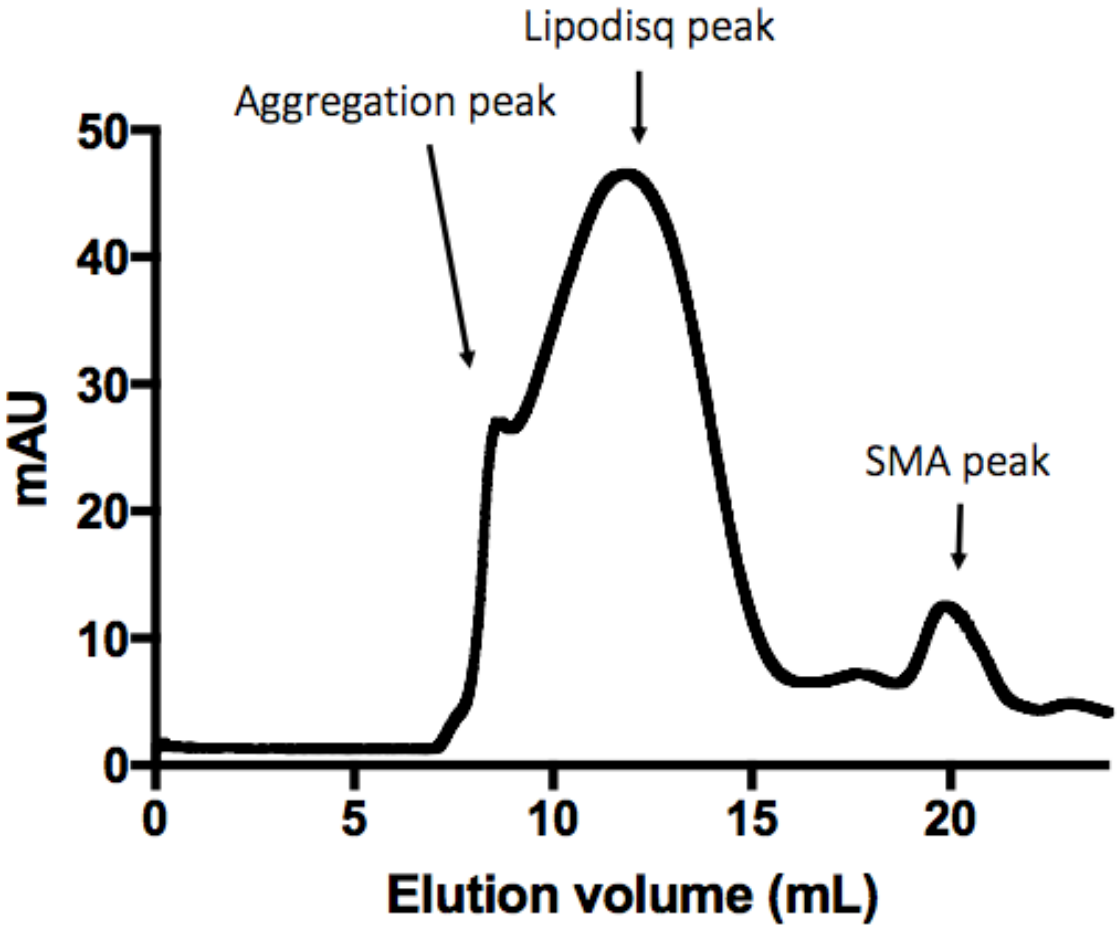

Figure 2 Size-exclusion profile of D1-Lipodisq on a Superdex 200 Increase, where proteo-Lipodisqs elute between 8.5-13 mL. Free SMA elutes at 20-22 mL. Here the aggregation peak has been collected and not discarded since higher oligomeric states of the protein could be present in the void volume of the column.

A typical SEC elution profile is shown in Figure 2. Here, all the samples from the elution volumes 8.5 mL to 13 mL were collected, including the void volume peak, only the late fractions containing the free SMA were

discarded. DRs have been observed in several different oligomeric states as monomer, dimers or tetramers[14,53–55] and as the oligomeric state would have an impact on the final Lipodisq size we chose not to exclude any fractions at this stage.

Once the excess polymer had been removed, the samples were purified by IMAC using a HisTrap column. Samples were recirculated through the column, as a single pass yielded only a small amount of receptor due to the large size of the Lipodisq, impeding the interaction between the metal ion and the HisTag[34]. A low flow rate and several passes through the column accentuate the interaction between the ion metal and the HisTag as shown by Pollock and colleagues[34,56]. The temperature at which the purification was performed (4°C) was again chosen to ensure protein stability. Elution of the protein was performed by increasing the imidazole concentration in steps of 25 mM, 50 mM, 100 mM and 250 mM (Figure 3). The 25 mM imidazole clean-up step elutes any non-specific protein bound to the column, hence the multiple bands present in the corresponding SDS-PAGE gel lane in Figure 3. The 50 mM imidazole step was intended to initiate the elution of the protein, but no gel bands were observed corresponding to the protein of interest. At high imidazole the protein elutes with high purity as shown in the gel lanes on the right in Figure 3. The yield of the purification was calculated using BCA assay and estimate to be 0.255 mg/L of culture. This is somewhat similar to the yield obtained in the literature[56,57]. Silver stain was used to ensure that the protein had not co-eluted with any impurities. We speculate that the high molecular weight species present is a dimer of the D1 protein. Next, to confirm that no other protein was present in our sample, western blot and proteomics experiments were performed.

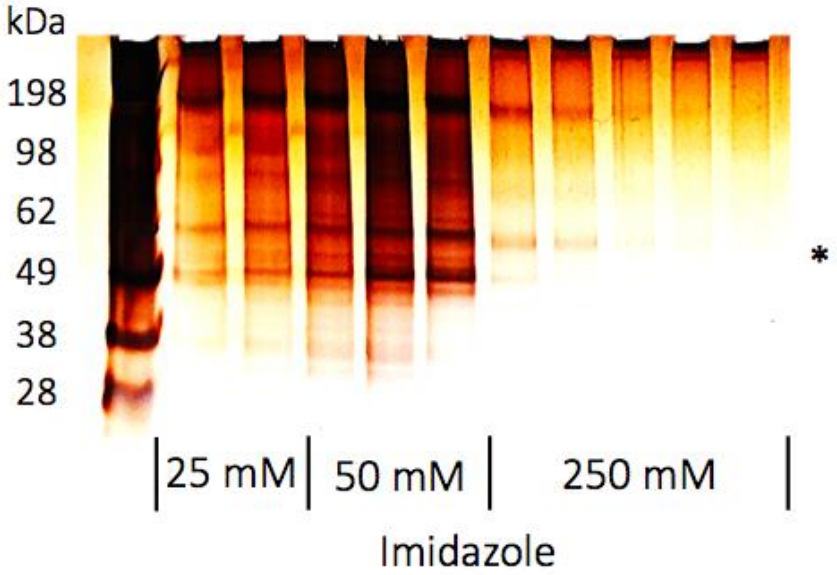

Figure 3 SDS-PAGE gels from D1 Lipodisq IMAC purification. Protein of interest (marked with *) is typically eluted at concentration higher than 50 mM imidazole and is relatively pure at high concentration of imidazole.

The presence of the protein was verified by western blot analysis as shown in Figure 4. For the D1-LQ sample, enhanced chemiluminescence (ECL, see Supplementary information for experimental details) was performed as the quantity of purified receptor was low (< 1 µM). An anti-His antibody and the D1 specific Fab antibody fragment both reveal a band at the expected molecular weight of the protein, confirming that the protein has been purified.

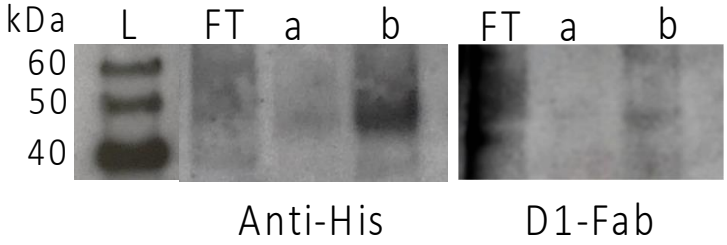

Figure 4 Western blots for D1 Lipodisq samples. Anti-His and D1-Fab reveal the presence of the D1 receptor in the sample purified after IMAC at the expected molecular weight of the receptor and correlates with the band observed in the SDS-PAGE. FT: Flow through; a: 25 mM imidazole fractions; b: 250 mM imidazole fractions

Due to the small amount of receptor purified, to further confirm that D1 was present in the Lipodisqs after the IMAC purification by an orthogonal method, proteomics experiments were performed by LC/MS/MS (Table 1). Three peptide fragments were identified as unique to D1, being

derived from the C-terminus, the intracellular loop 3 and the extra-cellular loop 2 confirming that D1 was purified in the Lipodisq via SMA solubilization.

Table 1 Peptides identified from the proteomics experiments on D1 Lipodisq samples.

Unique peptides of hs-D1 sequence identified	
EEAAGIARPLEK	C-terminus
NCQTTTGNGKPVECSQPESSFK	Intracellular loop 3
AKPTSPSDGNATSLAETIDNCDSSLSR	Extra-cellular loop 2

3.2 Radio-ligand binding assays

Before assessing the activity of the receptor, the folding of the protein was studied using circular dichroism (Figure S1). The CD data reveal that the D1 fold is retained in the Lipodisq. The affinity properties of the D1 protein reconstituted into LQ was confirmed by radio-ligand binding (Figure S2). The binding affinity of the protein in native HEK293f cell membranes was established by using the specific D1 antagonist, SCH23390 and the level of non-specific binding was assessed using (+)-butaclamol[58,59]. The data (Figure S2) reveals that the protein is able to bind ligands in both the native membrane and in Lipodisqs. Importantly, the binding affinity and receptor density (B_{max}) of SCH23390 was similar for D1 Lipodisqs and D1 HEK membranes, which confirms that the protein binding affinity is maintained during the SMA-LQ extraction from the membrane.

Similar results have been observed for the adenosine receptor (A_{2a})[41], which was extracted from HEK cells using SMA. Jamshad et al. showed that A_{2a} in SMALPs display similar binding affinity to the receptor from HEK membranes. This observation suggests that SMA is a suitable new alternative for membrane protein purification enabling the study of the different GPCR signalling pathways in a more native environment than the detergent setting. To emphasise how crucial the protein-lipid environment is for ligand binding or protein signalling, it has been demonstrated that dopamine receptors, upon interaction with certain drugs such as cocaine, are able to translocate to a different lipid environment[60,61]. Thus, the ability to directly extract proteins in their preferred local lipid composition using SMA Lipodisqs may offer significant advantages over current approaches.

3.3 Microscale thermophoresis experiments reveal the interaction of neurotensin peptide to D1 receptor in Lipodisqs

Several *in vivo* studies have highlighted the possible interaction between neurotensin and dopaminergic system[62]. It has been shown that the neurotensin peptide (NT) can modulate dopamine release in various brain structures[63–68], and that NT and dopamine (DA) colocalize in certain compartments of the brain[69,70]. In addition, NT can regulate the dopaminergic transmission in certain tissues[62,68].

Herein, we have demonstrated using microscale thermophoresis that, *in vitro,* neurotensin is able to bind specifically to the purified D1 embedded into Lipodisqs. We hypothesize that the stable and native lipid environment provided by the Lipodisq produces the ideal environment for the interaction between the receptor and the neuropeptide to be studied.

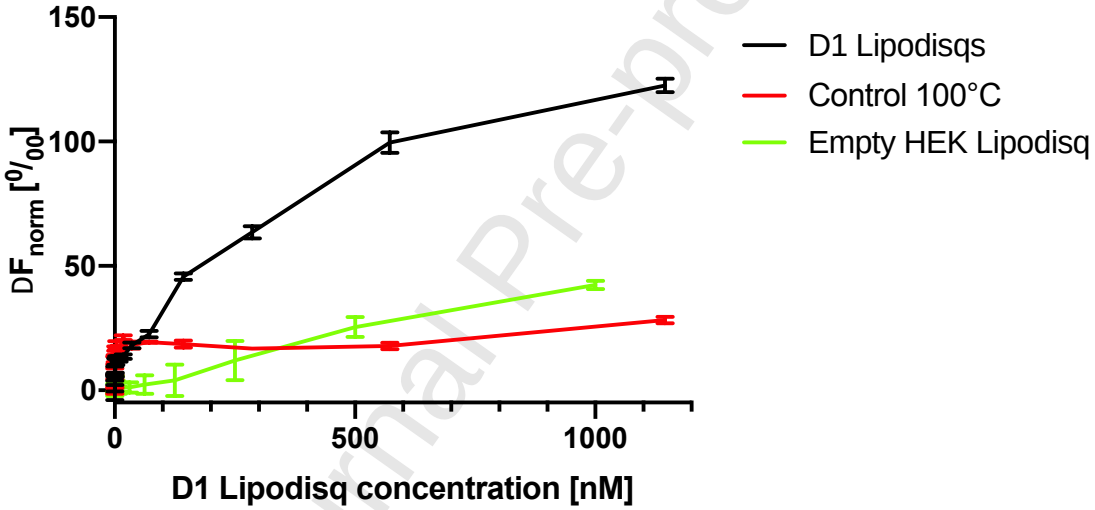

Figure 5 MST titration assays. Binding affinity experiments were conducted using labelled NT (NT-Cy5). The affinity of NT-Cy5 for D1 Lipodisqs was > 300 nM. Denatured D1 Lipodisq and Lipodisq formed from HEK cells lacking D1 (red and green curves respectively) did not show binding to NT. Error bars represent the standard error of n=3 measurements.

As it can be observed in Figure 5, the interaction between NT and D1 in Lipodisq is clear. Moreover, the binding is only due to the presence of D1 receptor in the discs since the two performed controls evidence that neither the lipids nor the polymer or other proteins present in the HEK plasma membrane interact with NT (red and green curve in Figure 5). Unfortunately, we were not able to reach receptor saturation therefore we cannot reliably calculate the affinity of NT-D1 receptor binding. However, we can estimate that it is higher than 300 nM. Although this value is at least 30 times lower than the affinity of dopamine to D1

receptor (K_D=9 nM)[8,62] and also lower than the affinity of NT for other neurotensin receptors (K_D=0.1-10 nM)[62,71] it is none-the-less in a biologically relevant range. Hence, to the best of our knowledge, we report the first evidence of a specific interaction *in vitro* between NT and D1 receptor.

4. Conclusion

We report for the first time the use of SMA copolymer for extraction and purification of a wild-type human GPCR from its native lipid environment via detergent-free Lipodisq formation. Proteomics and western blot experiments confirm the presence of the receptor after purification, while radioligand binding confirms that the binding affinity of the receptor is not affected after SMA solubilization. Importantly, we showed for the first time the existence of binding of the neurotransmitter neurotensin to D1 receptor, an interaction that might have important biological implications as dopamine receptors have been shown to form heterodimers with neurotensin receptor 1 (NTS1)[72–74]. Our work paves the grounds for the purification and characterisation of wild-type GPCRs in their functionally relevant lipid environment. This is of key importance in structural biology and drug screening, ultimately unravelling relevant biological questions.

Acknowledgments

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Highlights

- We report for the first time the extraction and purification of a wild-type human GPCR from its native lipid environment via detergent-free Lipodisq formation.
- It is the first time that microscale thermophoresis is used to perform ligand binding for protein embedded in Lipodisq.
- We prove for the first time the existence of binding of the neurotransmitter neurotensin to D1, an interaction that might have important biological implications.