博士論文

Cell-Free Synthesis of the Human Membrane Protein: The Chemokine G-Protein Coupled Receptor

（ヒトケモカイン受容体の無細胞合成の研究）

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Cell-Free Synthesis of the Human Membrane Protein: The Chemokine G-Protein Coupled Receptor

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A Doctoral Dissertation Submitted to

The Department of Computational Biology and Medical Sciences

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I. ABSTRACT

The application of the cell-free expression systems to study membrane proteins which are the prime targets of drugs is relatively a new approach. Currently, an in vitro protein synthesis systems from different sources are employed to overcome the challenges of conventional in vivo approaches for heterologous expressions. The Protein Synthesis Using Recombinant Elements system (PURE system), which is an *E. coli* based cell-free system, offers a great advantage due to its open and versatile nature. Hence, artificial lipid mimetics such as nanodiscs or liposomes, micelles, detergents and chaperones can be incorporated during the in vitro synthesis of target membrane proteins. In this study, we investigated the synthesis of active chemokine G-protein coupled receptors (GPCRs) capable of binding the agonist ligand and made a preliminary study for future structural elucidation.

On account of their pharmacological significance and also their pivotal role in human physiology, two human chemokine GPCRs, CX_3CR1 and CCR5 were expressed by the PURE system in the presence of artificial lipid mimetics such as nanodiscs (ND) or giant unilamelar vesicles (GUVs). The abnormal expression of these receptors were associated with various disease such as HIV-AIDS, cancer, asthma and cardiovascular disease. The structure of CCR5 is already solved but not for CX_3CR1. Hence, CX_3CR1 was expressed in the presence and absence of nanodiscs and a productivity of 1.3 µM and 1.8 µM as a total (T) protein was obtained respectively. Similarly, a productivity of 0.24 µM and 0.9 µM as a total amount of CCR5 was synthesized in the absence and presence of nanodiscs respectively.

The solubility of the receptors was dramatically higher in the presence of nanodiscs with a solubility of 92% and 74 % for CX_3CR1 and CCR5 respectively. Moreover, the insertion of rubredoxin (Rb) to CCR5 enhanced the productivity by twofold as compared to the wild CCR5. However, the solubility was improved only slightly.

Thus, the productivities of chemokine receptors are sufficient for biochemical and/or biophysical analysis. With further optimization of the productivity, the yield will be sufficient for structural analysis too. In addition, nanodiscs play a substantial role in enhancing the

solubility and also stability of the receptors. Our data has also shown that stabilization of receptors by rubredoxin greatly increased the stability and productivity.

To confirm the secondary structure formation, the receptors were synthesized in a PURE system using a micelle to avoid the interference of the scaffold protein as it contains α-helix. Both CX_3CR1 and CCR5 showed a typical characteristics of α-helix proteins with a minima at 208 ad 222 nm wavelengths. The CD spectra showed that the α-helix content of CX_3CR1 and CCR5 are 40% and 49.5% which is in agreement with a seven transmembrane (7TM) proteins. To further confirm the functionality of the receptor synthesized by the PURE system, we analyzed the ligand binding of CX_3CR1 by surface plasmon resonance.

For the ligand binding, we synthesized CX_3CR1 in the presence of either nanodiscs or micelles, allowed to interact with the agonist ligand and measured the binding affinity constant using SPR. To determine the binding affinity, we designed two approaches. The first approach involves immobilization of His-tagged CX_3CR1 in nanodisc on to a CM5 sensor chip pre-immobilized with anti-His antibody. In this approach, the agonist ligand was injected in an increasing concentration and a binding affinity constant of 27.4 nM was obtained. The second approach involves the synthesis of the CX_3CR1 ligand, CX_3CL1, containing strep-II tag by the PURE system and immobilized to a CM5 sensor chip pre-immobilized with StrepMAB Immob antibody. In this approach, a binding affinity constant of 39.9 nM was obtained when PURE synthesized CX_3CR1 in micelle was injected at increasing concentration. The binding affinity constants indicate that CX_3CR1 synthesized by the cell-free system (the PURE system) is active.

To probe the localization of the receptor, CX_3CR1 fused with sfGFP was synthesized by the PURE system in the presence of GUVs and the localization was analyzed by confocal microscopy. As a control, we synthesized sfGFP alone and compared the localization. Since, CX_3CR1 is a membrane protein, it localizesd on the membrane unlike the soluble sfGFP which localized in the lumen of the GUV indicating proper localization of CX_3CR1.

In order to use PURE synthesized CX_3CR1-nanodisc complex for structural analysis, we purified the complex by size exclusion chromatography and examined the homogeneity of

the complex by negative staining using transmission electron microscopy. Ashift in an elution profile was observed for CX_3CR1-nanodisc complex compared to empty nanodiscs which shows integration of the receptor into the nanodisc. The complex was also appeared as homogeneous which is necessary for structural analysis of the receptor in nanodisc.

In conclusion, our results showed that the cell-free system is a promising approach to study chemokine GPCRs and other classes of GPCRs. The synergy of the PURE system with the nanodisc technology will be substantial to study ligand binding and other functional assays. Furthermore, the cell-free system will play a key role in structural elucidation of GPCRs in nanodisc using the emerging Cryo-EM.

II. List of abbreviations

7TM	Seven transmembrane
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
GPCR	G-protein coupled receptor
IPTG	Isopropyl b-D-1thiogalactopyranoside
MSP	Membrane scaffold protein
NMR	Nuclear magnetic resonance
NTA	Nitrilotriacetic acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
PMSF	Phenylmethanesulfonyl fluoride
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt)
DLS	Dynamic light scattering
CM5	Carboymethylated dextran
ND	Nanodisc

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1. GENERAL INTRODUCTION

Membrane proteins are the major drug targets constituting of more than 50% of the current drugs in the pharmaceutical industry [1–4]. They are associated with the lipid bilayer and modulate the functions of the membrane by regulating the bidirectional flux of ions and molecules. Similarly, the functions of membrane proteins are modulated by the composition of the lipid bilayer which regulates the activity and affect membrane proteins [5,6]. With this interdependence and their potential in drug discovery, it is necessary to study the different classes of membrane proteins. Membrane proteins are generally classified into three categories such as integral, peripheral and anchored which transverses the membrane, attached to the membrane and, attached to the membrane through lipids anchored to the membrane respectively.

Among the integral membrane proteins such as receptors, ion channels and transporters, the G-protein coupled receptors (GPCRs) play a substantial role in cellular and physiological functions. Currently there are more than 800 GPCRs including olfactory and non-olfactory receptors in the human genome. Approximately, 350 of the receptors are non-olfactory receptors with known and unknown ligands. Receptors with unknown ligands are known as

GPCRs are the largest class of integral membrane proteins with approximately 800 GPCRs of which ~3500 are non-olfactory GPCRs, ~400 olfactory and ~ 130 orphan GPCRs that lacks endogenous ligand are encoded in the human genome [7,8]. Various physiological functions are regulated by GPCRs and they represent the major drug targets in the fight against disease. The development of drugs targeting GPCRs is a multibillion industry and currently it represents approximately 25-50% of pharmaceutical drugs in the market. However, despite their significance, only less than 10% GPCRs are targeted due to lack of knowledge of their function and structure. Among these classes of GPCRs, the chemokine receptors are the major targets for drugs. Although the structures of some chemokine GPCRs are solved, the structures of majority of these class of receptors are still not yet solved and demands continuous efforts to study their function-structure relationship.

GPCR structures were solved predominantly by X–ray crystallography and NMR. However, structural determination demands larger quantities of GPCRs which is difficult to achieve in a functional and ligand-binding state through heterologous expression as these class of membrane proteins are expressed in insufficient amount in the cell. Currently, the Escherichia coli (*E.coli*), yeast (*P. pastoris*), insect/baculovirus (Sf9) and mammalian cells (HEK293 and CHO cells) are commonly employed for heterologous expression of GPCRs regardless of their limitations. For example, *E.coli* based expression, lack of post-translational modification and the mismatch in lipid type and composition of the lipid bilayer affects the ligand binding properties of some GPCRs. Nonetheless, the *E.coli* system offers a great advantage for uniform isotope labeling in addition to their low cost and ease of use. Similarly, non-uniform glycosylation and lipid composition in yeast, higher amount of unsaturated lipids and low content of cholesterol in baculovirus and time-consuming and high cost of expression in mammalian cells are some of the limitation of the systems. A novel in vitro system based on a cell-free system is recently emerging as a new platform to express membrane proteins including GPCRs [9,10].

1.1.Membrane Proteins (MPs)

Membrane proteins are the largest group of proteins which interacts with lipid bilayer and classified into three main categories as integral, peripheral and lipid anchored (Figure 1) [7,11]. The integral membrane proteins constitute the largest class of membrane proteins which play vital roles for cell-to cell communication, intracellular signaling, ion transport and energy production. They interact and transverse the lipid bilayer as a single or multi transmembrane helix. The integral membrane proteins include G-protein coupled receptors (GPCRs), ion channels and transporters. However, the peripheral membrane proteins are anchored to the membrane and do not transverse the membrane. The last class of membrane proteins, lipid anchored membrane proteins, anchored to the membrane through the attachment to the polar head groups of the phospholipid. Among the integral membrane proteins, the GPCRs are

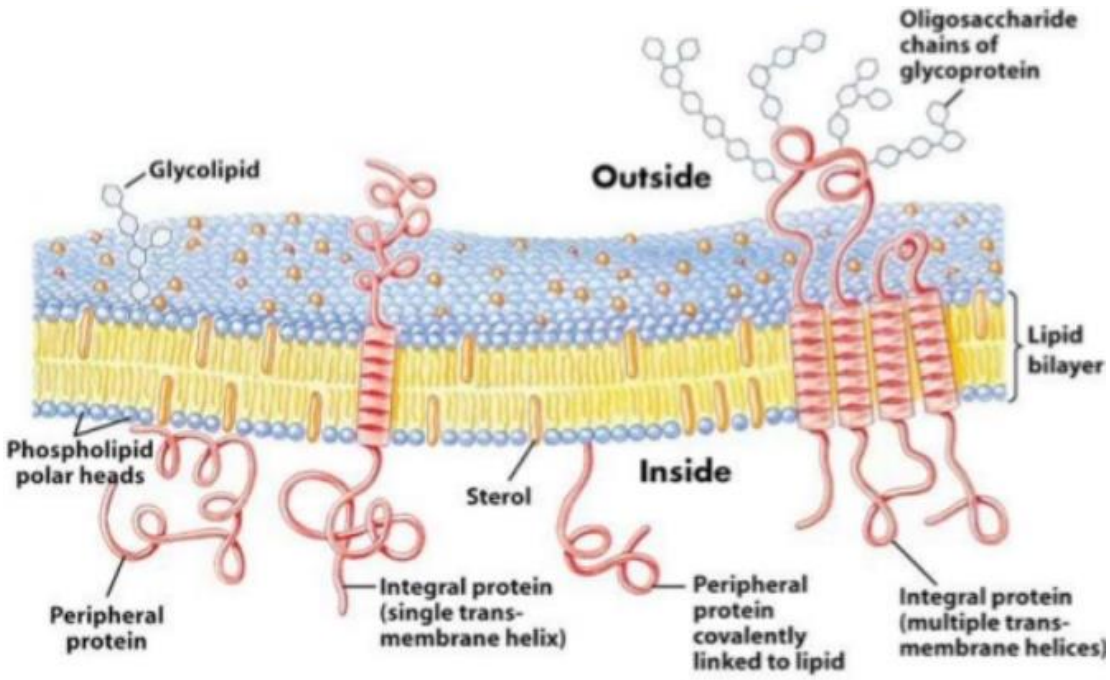


Figure 1: The classification of membrane proteins based on their interaction with the membrane. (Adapted from Nelson and Cox, Lehninger Principles of Biochemistry 4th edition)

1.2.The G-protein coupled receptors (GPCRs)

The GPCRs represent the largest class of integral membrane proteins with approximately 800 GPCRs are encoded in the human genome [7,8]. Among these GPCRs, more 350 GPCRs are non-olfactory receptors and modulated by small peptides and proteins. The other GPCRs are olfactory GPCRs which includes the sensory, olfaction, taste and light perceptions. The GPCRs are characterized by a seven transmembrane (7TM) helix which is buried inside a hydrophobic core of the lipid bilayer with the N and C terminus protruding outside (extracellular) and inside (cytoplasmic) side respectively. It also contains three extracellular and three intracellular loops which are important for ligand binding and signaling respectively [12]. Although there are receptors with 7TM, GPCRs are unique in a way that they signal through G-proteins (Figure 2).

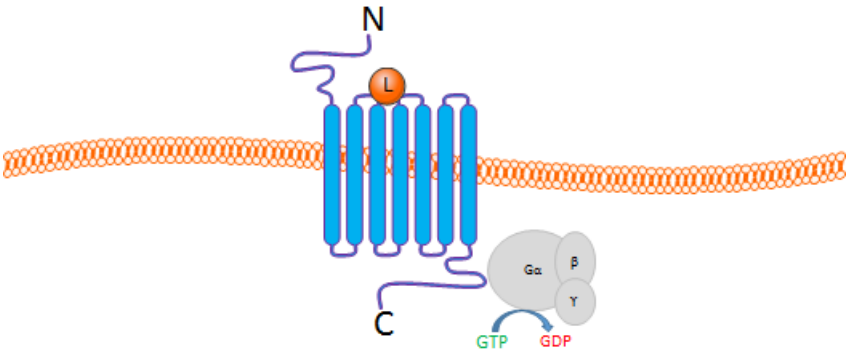


Figure 2: Schematic representation of GPCRs in a lipid bilayer and their association with G-proteins.

Based on their sequence homology, GPCRs are classified into five major families and several subfamilies. These include the Glutamate, Rhodopsin, Adhesion, Frizzled/tast2 and Secretin families and abbreviated as GRAFS (Figure 3). The rhodopsin family which is also referred as class A GPCRs are the most abundant GPCRs. The class A GPCRs varies in their primary amino acid sequences and shows different ligand preference and specificity. However, class A GPCRs generally possess short N terminus and a conserved sequence in the transmembrane (TM) such as the DRY motif in the 3TM, CWxP in the 6TM and NPxxY in the 7TM. The GPCRs are further classified as α, β, ϒ, δ subfamilies which binds biogenic

monoamines, small peptides, both peptides and lipids and odorants respectively. For example, histamine, endothelin, chemokine and olfactory receptors are grouped under α, β, ϒ, δ subfamilies.

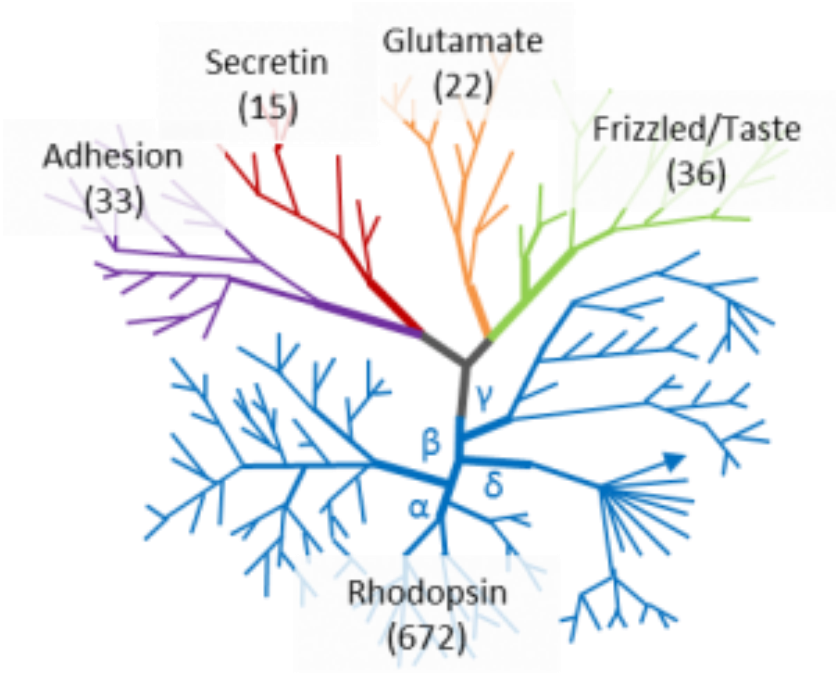


Figure 3: Sequence homology based classificication of GPCRs. Adapted from The GPCRdb database.

1.3.Chemokines and chemokine receptors

Chemokines are soluble and low molecular weight (8-12 kDa) chemotactic cytokines that play a crucial role in the immune system [13]. Chemokines are involved in the development of organs and tissue repair. In addition, they direct the migration of immune cells between different organs during homeostasis and recruitment of pathologic immune cells. To date, approximately 44 human chemokines are known to bind to 21 human chemokine receptors [14]. Based on the number of amino acids (designated by X) between the first and the second cysteine, chemokines are categorized into four classes such as C, CC, CXC and CX_3C (Fig 4). The C chemokines share homology with CXC and contain only two cysteines unlike other chemokines. Whereas, the CC chemokines contain four cysteines and they lack an amino

acid between the first two cysteines. However, the CXC and CX_3C chemokines contain one and three amino acids between the first and the second cysteines respectively.

Chemokines can also be classified based up on function and regulation into inflammatory and homeostatic. The inflammatory chemokines include the responses of the T-cell differentiation responses such as innate and adaptive immune responses. The homeostatic chemokines are mainly involved immune surveillance such as lymphocyte trafficking.

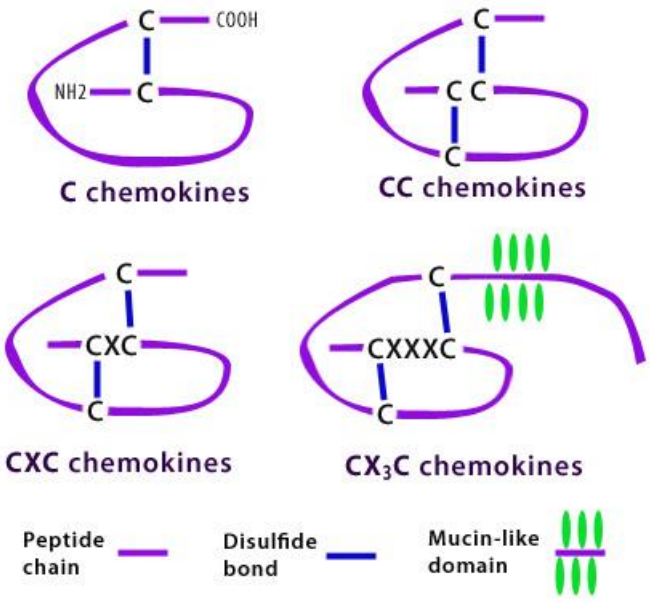


Figure 4: Classification of chemokines based on their intervening sequence in the first two cysteines.

Chemokine receptors are the largest receptors of the ϒ subfamily in the rhodopsin family of a seven transmembrane spanning G-protein coupled receptors (GPCRs) that signals through intracellular heterotrimeric G proteins. They are mainly expressed in leukocytes and involved in various pathologies. The chemokine receptors activated through pertussis toxin (PTX) sensitive Gi-type G proteins could sometimes bound β-arrestins and inhibit the G protein activation a process known as desentization and involved in receptor internalization. Based on their signaling, chemokine receptors are classified into 20 signaling and 5 non signaling

receptors. Chemokine receptors which signal independent of G proteins are known as atypical receptors that lacks an intracellular motif critical for signaling for signaling receptors. The atypical chemokine receptors are mainly involved in regulating the chemokine gradient.

The chemokine receptors are also classified into four classes based on the chemokine ligand they bind. These include the C, CC, CXC and CX_3C chemokine receptors which binds C, CC, CXC and CX_3C chemokines respectively. However, the binding of a chemokine to the receptor is either specific or shared. As a result, chemokine receptors are expressed in different level which varies in specificity and promiscuity. For example, CCR5 binds two three different chemokines (Rantes, MIP-1α and MIP-1β) but CX_3CR1 binds only to CX_3CL1 (fractalkine) [15,16].

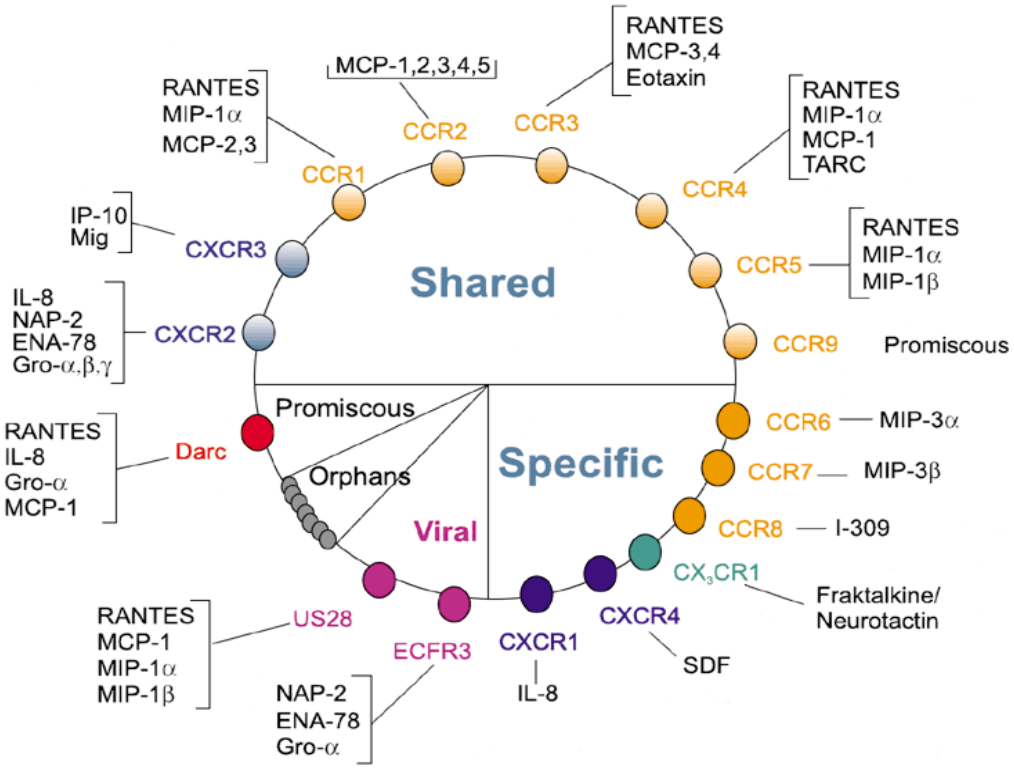


Figure 5: Association of chemokine ligands with specific and shared chemokine receptors

Some viruses also encode for chemokines and chemokine receptors which arises through evolution from human genome hijacking and expressed in the host cell after infection. The expression of viral encoded chemokine GPCRs affect

Chemokines interaction with their receptors are important in maintaining the immune cell homeostasis. For example, the interaction of CXCL12 and CXCR4 plays a key role in the maintenance of hematopoietic stem cells and developing of immune cells in the bone marrow (BM). The release of neutrophils from the BM, enter into the blood stream and promote their migration

1.4.The role of chemokines and chemokine receptors in disease

Most chemokine receptors serve as a coreceptor for the entry of HIV virus in macrophages and CD4+ T-cells [17,18]. The two structurally solved chemokine receptors, CCR5 and CCR4 were studied as a receptor for the virus. Tang *et al*, has solved the structure of CCR5 in complex with a small antagonist molecule that is known to block the entry of the virus. In addition to serve as coreceptor for HIV/AIDS, chemokine receptors are also involved in various pathological disease such as cancer metastasis, arteriosclerosis, inflammation and cardiovascular diseases. Therefore, understanding the function and structure relationship of chemokine receptors and their interaction with their agonist ligands dictates a significant advantage to develop drugs against such disease.

1.5.Recombinant expression and purification of membrane proteins

The eukaryotic and bacterial expression hosts are solely used to study the function and structures of membrane proteins. Each system has their own advantages and limitations as an expression system for the difficult-to-express membrane proteins such as GPCRs which are naturally expressed less abundantly. The cell-based in vivo method is sometimes affected by host cell toxicity, aggregation and mis-folding [9,10]. Though the bacterial based in vivo approach is easy for scaling up the expression, codon optimization is necessary to increase productivity. Furthermore, eukaryotic based in vivo expression hosts are currently the most widely used systems for most of membrane proteins as it is an ideal system for post translational modification and proper folding.

Most of the membrane proteins are extracted and solubilized using detergents from the membrane. However, detergents are detrimental to the activity and stability of membrane proteins. To minimize the detergent contact and to produce a membrane protein in a controlled condition, currently, an in vitro cell-free systems in conjunction with artificial lipid mimetics are widely employed. Among the different types of cell-free systems originating from different sources, the Protein Synthesis Using Recombinant Elements system (PURE system) is ideal due to its improved controllability.

1.6.The cell-free expression system

The cell-free systems are a bottom-up synthetic biology approach which are mainly used as alternative method of in vivo methods of protein expression. These includes the wheat germ, bacterial cell extract lysate, rabbit reticules and the reconstituted recombinant proteins (the PURE system). The cell-free system is an ideal system to express toxic proteins and for uniform isotope labeling [9,19,20].

The cell-free system is widely used for various soluble proteins. However, their use for membrane proteins is mainly limited by the hydrophobic nature of membrane proteins. Therefore, addition of artificial lipid mimetics such as liposomes and lipid nanodiscs is mandatory for both co-translational and post-translational reconstitution systems. Due to heterogeneity of in orientation of membrane protein insertion and accessibility of only one terminus for ligand binding, liposomes are less preferred for functional assay. As an alternative to liposomes, nanodiscs are extensively used. Currently, a SMALP system based on the styrene maleic anhydride system is used to directly solubilize membrane proteins from their membrane and to retain them in a nanodisc system containing native lipids. However, this system requires expression of target membrane proteins in vivo.

1.7.The PURE system

The PURE system (Protein synthesis Using Recombinant Elements) is an *E.coli* based on reconstituted cell-free translation system [21] composed of only important transcriptional and translational factors which are purified from bacterial extract. Though the PURE system is capable of expressing MPs in the presence of detergent, chaperones, liposomes and nanodiscs, it was not explored well as it was used to synthesize soluble proteins.

The greatest advantage of using the PURE system is that the reaction components can be manipulated as per the desired function. It is also an important system to express unnatural amino acids and toxic target genes. The PURE system can also be scaled up easily to a continuous system for higher productivity. Thus, the system is amenable for optimization of membrane protein productivity.

1.8.The nanodisc (ND) System

Nanodiscs are discoidal appolipoproteins which are first developed by Sligar and co-workers. Now a days, nanodiscs become the choice for studying membrane proteins as it enhances stability and activity [22–24]. The advantage of using nanodiscs include stability, solubility, monodisperse and more importantly the N and C terminus of the membrane proteins in lipid nanodisc are accessible for ligand binding. Various membrane proteins were reconstituted into nanodiscs post-translationally and co-translationally.

Recently, nanodiscs are employed for structural studies of membrane proteins using Cryo-EM. The larger size of nanodiscs offer a greater advantage to increase the membrane protein-nanodisc complex and enables us to easily overcome the size limitation of Cryo-EM.

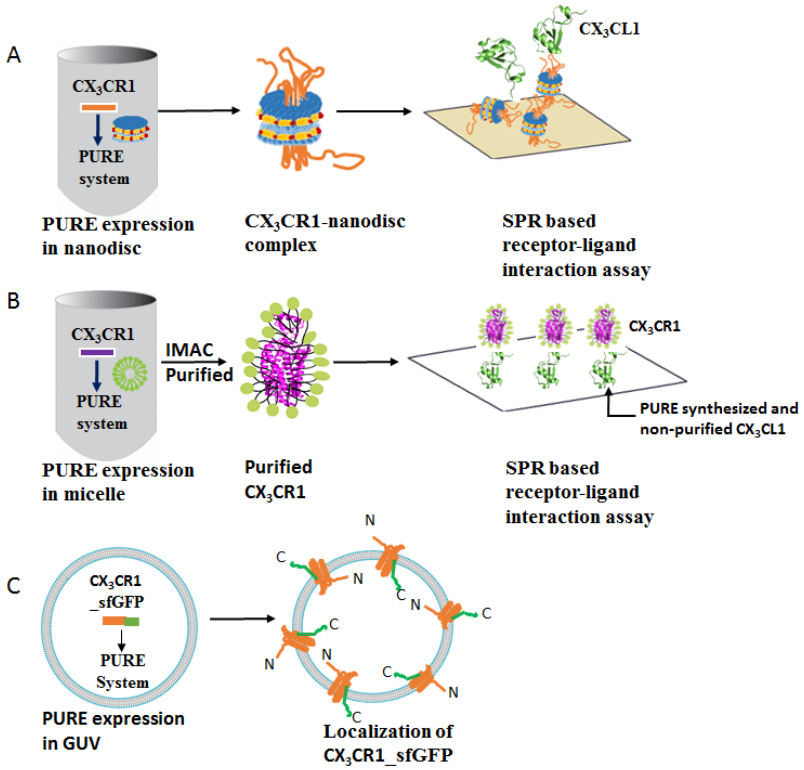


Figure 6: Schematic representation of the cell-free expression of GPCRs. CX_3CR1 was synthesized by the PURE system supplemented with lipid nanodiscs (**A**), micelle (**B**), or CX_3CR1-sfGFP synthesized inside GUV (**C**).

1.9. G-proteins and their signaling mechanism

Heterotrimeric G-proteins consists of three complex subunits designated as Gα (~45KDa), Gβ (~37KDa), and Gϒ (~9KDa) in which GDP is bound to Gα in its inactive state. Upon the binding of a ligand to the N-terminus or ECL, the conformation of GPCRs are changed and is accompanied with the exchange of GDP by GTP. To date, 21 different Gα subunits, 6 different Gβ subunits and 12 different Gϒ subunits are known in humans [25].

All Gα except $G\alpha_t$ are susceptible for modification through palmitoylation. In addition, Gαi G-proteins are modified by myristoylation. For example, the chemokine receptor, CX_3CR1 couples to the inhibitory G-protein ($G\alpha_i$) and inhibits the formation of cAMP. The agonist ligand, fractalkine (CX_3CL1) binds to CX_3CR1 and activates the exchange of GDP for GTP. The GTP bound Gα will then bind to effector molecules such as AC and inhibits the formation of cAMP.

2. Project aim

GPCRs are the largest superfamily of integral membrane proteins targeted by 30-50% of current drugs on the market. Therefore, a novel approach to explore endogenous ligands and drugs has a substantial advantage to the pharmaceutical industry. Thus, a robust, efficient and productive approach is highly crucial to synthesize a stable and functional GPCRs in a lipid bilayer or micelle for functional and structural analysis.

The first aim of this work was to synthesize a functional chemokine GPCR in a bottom-up approach using a cell-free system supplemented with lipid bilayer mimetics or micelles

The second aim was to make preliminary studies to investigate the structure of chemokine GPCRs in nanodisc by cryo-EM.

3. Materials and Methods

3.1.Materials

Synthetic lipids such as 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG2000-DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA); Cholesterol from Nacalai tesque, Inc. (Kyoto, Japan); SM2 bio-beads from Bio-rad (Hercules, CA, USA); Penta-His antibody from Qiagen (Hilden, Germany); StrepMAB-Immo from IBA Lifesciences (Goettingen, Germany); CM5 and amine coupling reagents from GE Healthcare (Chicago, IL, USA).

3.2.Preparation of DNA Constructs

The cDNAs of human GPCRs, CX_3CR1 and CCR5, were a kind gift from Dr. Yutaka Suzuki (University of Tokyo). The sequence of CX_3CR1 was optimized for *E. coli* expression by Genscript whereas the sequence of human CCR5 was used without optimization. Plasmids and linear PCR products of both CX_3CR1 and CCR5 were constructed and used as a DNA template for protein expression. The PCR fragment of both CX_3CR1 and CCR5 were generated by two-step overlap PCR using a forward primer containing a T7 promoter and Shine Dalgarno sequence and a reverse primer with or without a His-tag. To enhance the stability of CCR5, a rubredoxin was inserted between amino acid residues Arg223 and Glu227 [26] to produce a stabilized CCR5 variant, CCR5-Rb, in which the template DNA preparation was the same as CX_3CR1 and CCR5. The expression plasmids of CX_3CR1, CCR5 and CCR5-Rb were constructed by infusion cloning of the DNA containing 6xhis tag and Tev recognition site preceded with a linker (DYDIPTT) at the N-terminus to pET28a vector digested with NcoI and XhoI restriction enzymes. For localization experiment, CX_3CR1-sfGFP fusion construct was made by fusing sfGFP to the C-terminus of CX_3CR1 DNA by overlap PCR and cloned to pET28a vector digested with EcoRI and XhoI restriction enzymes through infusion cloning. According to a previously reported method [27], the ligand fractalkine (CX_3CL1) containing strep-tag II at the N-terminus was amplified from pURE strept2 plasmid for immobilization and binding assay.

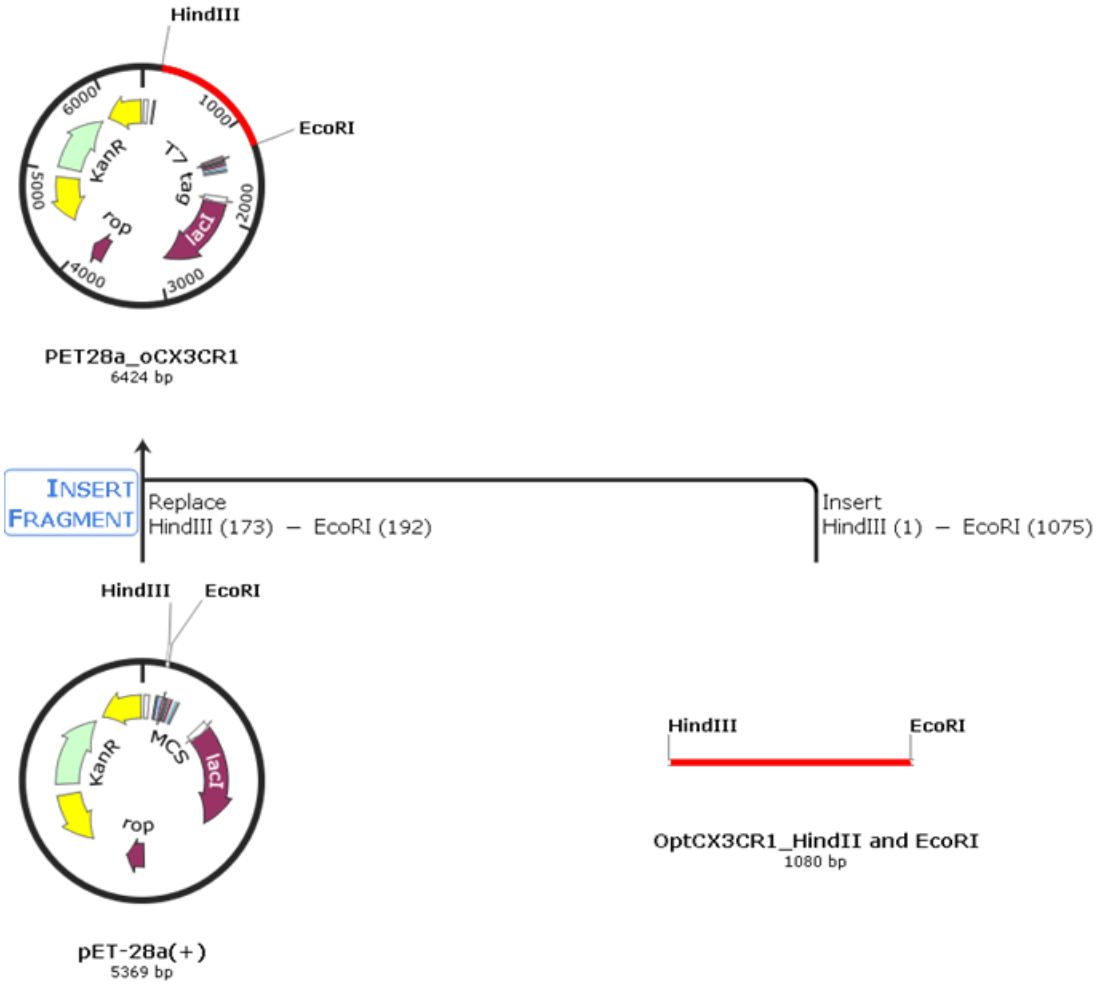


Figure 7: Cloning of CX3CR1 chemokine receptor in to pET28a vector.

3.3.Membrane Scaffold Protein (MSP) Expression

The expression and purification of MSP were carried out according to the established protocols with slight modifications [24]. Briefly, the expression host BL21 (DE3) containing either MSP1D1 (addgene#20061) or MSP1E3D1 (addgene#20066) plasmids were expressed in 3 L LB culture medium containing 50 µg/mL kanamycin at 37 °C with shaking and at OD_{600} 0.8–1, the culture was induced with 1 mM IPTG. The culture was further incubated for 1 h at 37 °C and for 3 h at 28 °C to prevent aggregation. The cell pellet was then collected at 8000× *g* for 15 min at 4 °C, washed with MSP buffer containing EDTA, flash frozen in liquid N_2 and stored at −80 °C. For purification, the MSP was thawed at room temperature for 30 min and suspended with lysis buffer (40 mM Tris/HCl, pH 8.0, 300 mM NaCl) containing 1% Triton X-100, protease inhibitor cocktail and DNase (0.5 mg for 5 gm cell pellet). The supernatant was then centrifuged at 30,000× *g* for 30 min at 4 °C to remove the cell debris and purified by IMAC affinity chromatography using Ni-NTA column pre-equilibrated with lysis buffer containing 1% Triton X-100. Subsequently, the column was washed with buffers A_{msp} (Lysis Buffer containing 1% Triton X-100), B_{msp} (Lysis Buffer containing 50 mM Na Cholate, 20 mM Imidazole), C_{msp} (Lysis Buffer containing 50 mM Imidazole). The MSP protein was later eluted with buffer D_{msp} (Lysis Buffer containing 400 mM Imidazole), analyzed by 15% SDS-PAGE and buffer exchanged with 20 mM Tris/HCl, pH 8.0, 100 mM NaCl and 0.5 mM EDTA in a PD-10 column. Finally, the fraction containing pure protein was collected, concentrated, aliquoted and flash frozen in liquid nitrogen at −80 °C until further use. The protein concentration was determined by absorbance at 280 nm with an extinction coefficient of ε_{280} = 21,000 M^{-1} cm^{-1} and ε_{280} = 29,400 M^{-1} cm^{-1} for MSP1D1 and MSP1E3D1 respectively.

3.4.Nanodisc (ND) Preparation

Nanodiscs were prepared according to previous reports with slight modification [23,28,29]. In brief, synthetic lipids POPC, POPS and Cholesterol were dissolved in chloroform and mixed at a ratio of 72%, 20% and 8% respectively. The mixture was then flushed with N_2 gas and dried overnight in a vacuum. The dried lipid film was solubilized by a nanodisc buffer (20 mM Tris/HCl, pH 7.4 and 100 mM NaCl) containing 100 mM Na cholate. To achieve complete solubilization, the mixture was vortexed followed by heating at 60 °C in a water bath until the lipid film completely dissolved. Finally, detergent solubilized lipid was mixed either with MSP1D1 or MSP1E3D1 at the respective molar ratio of 1:60 and 1:85 respectively and incubated at 4 °C. After 1 h of incubation, an adsorbent, SM2 bio-beads from Bio-rad (Hercules, CA, USA), was added to remove the detergent and to facilitate nanodisc formation. The reaction mixture was further incubated from 6 h to overnight and the Biobeads were removed by centrifugation at 130,000× *g* for 2 min using a 0.2 µm NANOSEP device from PALL Life Sciences (Port Washington, NY, USA). To remove the aggregates, the nanodisc was further centrifuged at 18,000× *g* for 5 min before loading to Superdex-200 10/300 from GE Healthcare (Chicago, IL, USA) for further purification.

3.5.PURE Synthesis of Membrane Proteins

A PURE system (PURExpress 2) prepared according to published protocols [30] and purchased from Genefrontiers (Kashiwa, Japan) were used for membrane protein synthesis. To facilitate correct disulfide bond formation, we used a modified PURE system in which the DTT was replaced by glutathione reduced (GSH). A GPCR was synthesized from 5 nM DNA template by the PURE system in the presence or absence of nanodiscs in a 20 µL reaction volume and incubated at 37 °C for 6 h. After the reaction, the synthesized membrane protein was aliquoted into 5 µL as a total fraction. The remaining 15 µL was centrifuged at 20,400× *g* for 10 min at 4 °C and the supernatant was collected. A sample loading dye was added at a 1:1 (*v*/*v*) ratio to the total and supernatant fraction and run on 15% SDS-PAGE. For radiolabeling based quantification, ^{35}S-methionine incorporation by the PURE system into the membrane protein was measured by BAS-5000 from Fujifilm (Tokyo, Japan) and the band intensity was quantified by Multi Gauge software (Tokyo, Japan).

3.6.Purification of Membrane Protein-Nanodisc Complex

After PURE synthesis, the reaction mixture was centrifuged at 18,000× *g* for 3 min at 4 °C prior to purification. The supernatant was mixed with Ni-NTA equilibrated with buffer A_{cplx} (20 mM Tris/HCl, pH 8.0, 150 mM NaCl) and incubated at 4 °C for 1 h with shaking. The column was washed with wash buffer B_{cplx} (buffer A containing 30 mM Imidazole) and the nanodisc-membrane protein complex was eluted with buffer C_{cplx} (buffer A containing 300 mM Imidazole. The elution fraction was run on SDS-PAGE to monitor the purity of the purification and buffer exchanged by dialysis with the elution buffer without imidazole, concentrated and quantified by A_{280} absorbance. For transmission electron microscopy, the elution fraction after IMAC purification was diluted 1:1 (*v*/*v*) in nanodisc buffer and further purified by size exclusion chromatography in a Superdex-200 10/300 column (GE Healthcare) using a nanodisc buffer.

3.7.Circular Dichroism (CD) Spectroscopy

Far-UV CD measurements were performed on J720 spectropolarimeter from Jasco (Tokyo, Japan) using a Teflon sealed 1 mm path length quartz glass cuvette from Hellma Analytics (Mullheim, Germany). A wavelength increment of 1 nm, a response time of 4 s, a scan speed of 20 nm/min parameters and a concentration of 0.15 mg/mL of CX_3CR1 and 0.11 mg/mL of CCR5-Rb were used during measurement. All the resulting spectra were buffer corrected.

Preparation of Membrane Proteins for CD Spectroscopy by Micelle Method

Mixed Micelles were prepared as described in Shinoda et al. [31] from 25 mg/mL brain polar lipid and 75 mg/mL of digitonin detergent and tip sonicated until the mixture becomes transparent. The micelle at 0.15 volume of reaction was added to a PURE based cell-free system and the reaction mixture was incubated for 4 h at 37 °C. After protein synthesis, the mixture was centrifuged at 100,000× *g* for 30 min at 4 °C and the supernatant was applied to Ni-NTA pre-equilibrated with buffer A (20 mM Tris/HCl, pH 8, 150 mM NaCl, 0.05% DDM and 0.002% CHS) and incubated at 4 °C for 1 h with gentle shaking. IMAC purification was carried out by washing the column in buffer B (buffer A containing 20 mM Imidazole) and the recombinant protein was eluted with buffer C (buffer A supplemented with 300 mM Imidazole). The eluate was then concentrated and buffer exchanged with buffer A by repeated dilute and concentrate method in a 10 kDa MWCO from Merck Millipore

(Tullagreen, Ireland). The concentration of the protein was finally quantified by A_{280} absorbance in nanodrop 1000 from Thermo Fisher Scientific (Waltham, MA, USA) and used for CD measurement.

3.8.Electron Microscopy of CX_3CR1-Nanodisc Complexes

IMAC purified CX_3CR1-nanodisc complexes were further purified by size exclusion chromatography and concentrated to 0.25 mg/mL using a 30 kDa molecular weight cutoff (Merck Millipore). About 2 µL of the CX_3CR1-nanodisc complex at 100 nM was deposited on a glow-discharged copper grid, incubated for 1 min and blotted away using a filter paper. Immediately after blotting, the grid was stained with 5 µL of 2% (*w*/*v*) uranyl acetate and blotted away after 30 s incubation at room temperature. The spotted sample was then dried at room temperature or using a lamp for quick drying and electron micrographs were recorded on H-7000 electron microscopy from Hitachi (Tokyo, Japan) operated at an acceleration voltage of 100 kV and at 30,000× magnification.

3.9.Giant Unilamellar Vesicle (GUV) Preparation

Giant unilamellar vesicle preparation was carried out according to established protocols with slight modification [13]. Briefly, POPC lipid or a mixture of POPC and PEG2000PE (9.75: 0.25 molar ratio) was mixed, at the concentration of 10 mM, with 500 µL liquid paraffin. The mixture was vortexed vigorously, flushed with N_2 gas and heated at 80 °C for 20 min. The heated lipid-paraffin mixture was vortexed until it gets cooled. The lipid-paraffin mix was flushed with N_2 gas again and subjected to water bath sonication for at least 30 min at 55 °C. The lipid-paraffin mixture was allowed to cool at room temperature and 300 µL of the mixture was transferred to a small glass tube. The inner solution mixture, composed of the PURE system, 200 mM sucrose and the template DNA in a 30 µL of reaction volume, was added at the bottom of the cooled lipid-paraffin mix. The emulsion was later formed by very brief and gentle pipette in-and-out. The prepared emulsion was then overlaid at the top of a 200 µL ice-chilled outer solution consisting of the PURE buffer without tRNA and 200 mM glucose in a 1:1 (*v*/*v*) ratio. The mixture was further kept on ice for 10 min and centrifuged for 30 min at 10,000× *g* at 4 °C. The precipitated GUV was collected by purging the Eppendorf tube at the bottom with 21 G × 1 ½-inch needle. RNase was added to the collected

supernatant at the concentration of 20 ng μL^{-1} and the GUV suspension was incubated at 37 °C for 6–8 h. Finally, the localization of the sfGFP fused CX_3CR1 was analyzed by confocal imaging. For sfGFP expression, the GUV was prepared from POPC and PEG2000-DOPE lipids.

3.10. Determination of Binding Constants by the Surface Plasmon Resonance (SPR)

SPR based ligand binding was carried out using Biacore T200 (GE Healthcare). Penta-His antibody (Qiagen) and strepMAB-Immo antibody (IBA Lifesciences) were immobilized on CM5 sensor chip using the standard amine coupling chemistry to capture His-tagged CX_3CR1-nanodisc complex and cell-free synthesized CX_3CL1 containing Strep-tag II respectively. In brief, flow cell one, which was used as reference and flow cell two of the sensor chip were activated for 7 minutes with a 1:1 mixture of 0.4 M EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) in water and 0.1 M NHS (N-hydroxysuccinimide) in water at a flow rate of 10 µL/min. About 9000 RU of Penta-His and 1000 RU of StrepMAB-Immo antibody at 50 µg/mL in 10 mM sodium acetate, pH 5.0 were immobilized for 7 min at a flow rate of 10 µL/min in a running buffer (HBS-P) containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% surfactant P20 for the nanodisc and micelle system respectively. To deactivate excessive reactive groups, the surfaces of both flow cells were blocked with a 1 M ethanolamine, pH 8.5. After 6 h of incubation at 37 °C, cell-free synthesized His-tagged CX_3CR1in nanodisc (~300 kDa) and CX_3CL1-tagged with Strep-tag II (8.5 kDa) were directly immobilized to flow cell two up to ~1500 RU and ~1000 RU respectively. For the nanodisc system, purified CX_3CL1 diluted in the running buffer was injected on both flow cells at a concentration of 5 nM, 10 nM, 20 nM, 40 nM and 80 nM and at a flow rate of 30 µL/min in the order of increasing concentration at 25 °C. The association and dissociation rate was set at 120 and 300 s, respectively. For the micelle system, PURE expressed CX_3CR1in micelle was diluted in the running buffer and injected to both flow cells with the same condition as the nanodisc system. A 10 mM glycine/HCl, pH 1.5 was used for surface regeneration. The K_D value was calculated with the Biacore T200 evaluation software version 3.0 using a 1:1 interaction model.

3.11. Confocal Microscopy

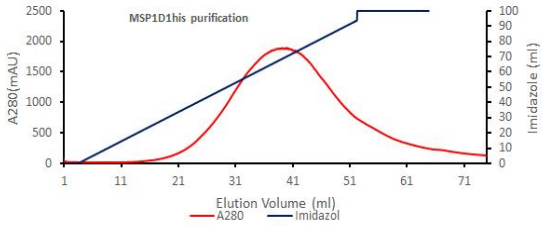
Confocal fluorescence microscopy was performed with an objective lens of 63×. After PURE synthesis, 5 µL of the sample was aliquoted and mixed with Nile red staining dye (100 µM) and both green and blue channels were selected for taking images. Upon taking the images, the sensitivity of the laser was adjusted optimum by Zeiss software.

4. Results

4.1.MSP purification

In order to integrate GPCRs into lipid bilayer mimetics known as nanodiscs, a scaffold protein which surrounds around the hydrophobic region of the bilayer has a significant advantage. The scaffold protein (MSP) enables us to control the number of lipid molecules and to control the sizes of the different nanodiscs. MSPs were purified by His-tag in the N-terminus using Ni-IMAC chromatography and the tag was cleaved by TEV protease after purification whenever it is necessary. Different variants of MSP were purified and in this study, we focused on two MSP variants, MSP1D1 and MSP1E3D1, which have a molecular mass of ~24 kDa and ~32 kDa (Figure). The purity of the fractions was confirmed by 15% SDS-PAGE and a discrete band of MSP1D1 corresponding to the expected band size of 24 kDa was obtained that will be further subjected to Tev cleavage of His-tag for nanodisc formation. (Figure 9).

A



B

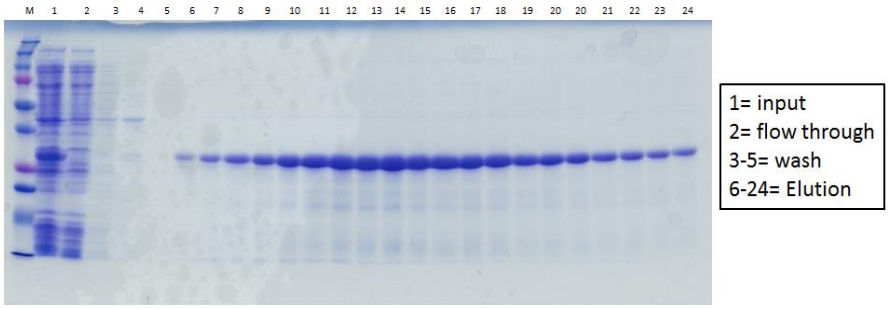


Figure 8: Purification of membrane scaffold protein (A) gradient elution of MSP1D1, (B) SDS-PAGE analysis of MSP1D1

To avoid the interference of the tag on MSP, the 7xhis tag at the N-terminus of MSP was removed by TEV which targets the TEV recognition site at the N-terminus after His-tag and the linker sequence. After incubation of MSP with TEV at room temperature, the supernatant was added to a column packed with Ni. While the non-cleaved MSP was trapped, the flow through and wash fractions were pooled, buffer exchanged and concentrated for storage or immediate use. For column wash, a buffer composed of 20 mM Tris/HCl, 150 mM NaCl and 25 mM Imidazole was used. After TEV digestion, it was possible to recover 50-60% of the initial protein.

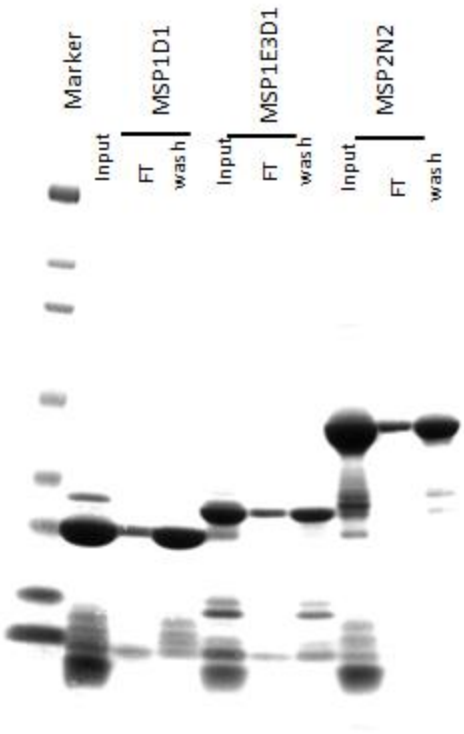


Figure 9: Tev protease digestion of membrane scaffold proteins. Three different MSP proteins, MSP1D1, MSP1E3D1 and MSP2N2, with a different molecular mass was digested by Tev.

4.2.Nanodisc Preparation and purification

In addition to membrane scaffold proteins, lipids were equally critical to make nanodiscs with the desired function. Recently, a plethora of lipids are available that can be used as a single lipid or as a mixture of lipids to make nanodiscs depending on the effect of the lipid on target protein. In this study, nanodiscs from MSP1D1 and MSP1E3D1 devoid of his tag and a mixture of lipids composed of POPC, POPS and cholesterol were formed at 4 ^{0}C by removal of Na-cholate detergent

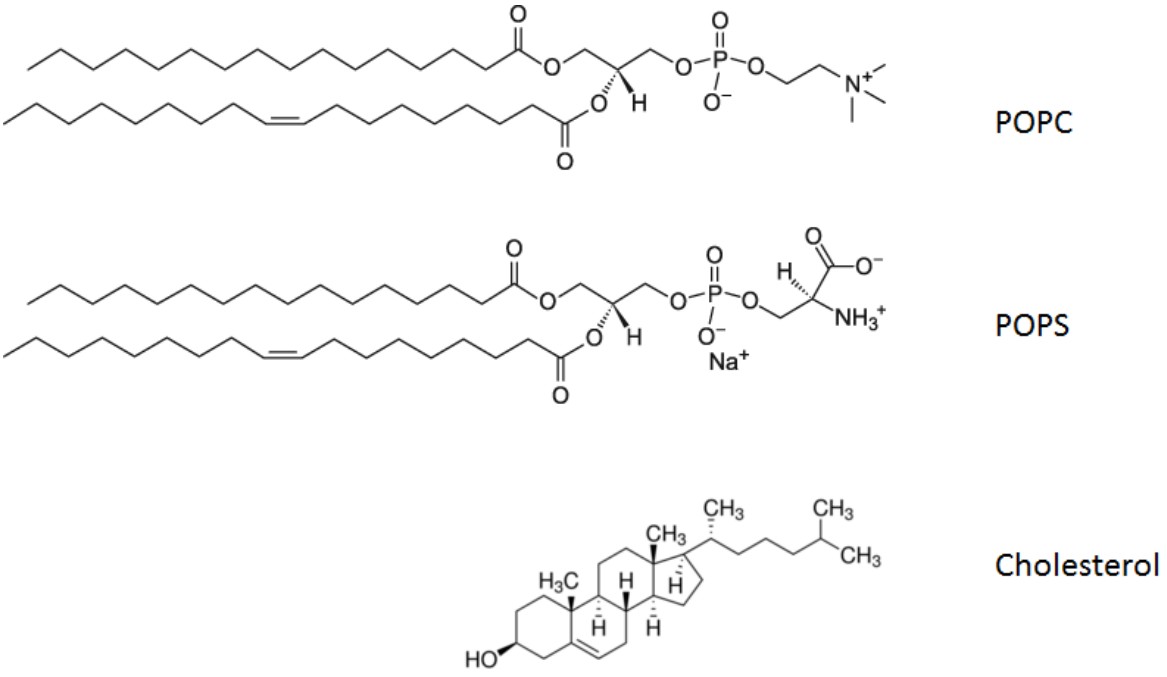


Figure 10: Synthetic lipids used for nanodisc formation (Adapted from Avanti Polar Lipids)

To obtain homogeneous nanodiscs, nanodiscs were separated from aggregates and free MSP by size exclusion chromatography using Superdex-200 10/300 to the level of the desired homogeneity. The expected size distribution of nanodiscs using MSP1D1 and MSP1E3D1 scaffold proteins are 9.7 nM and 12.8 nM in diameter respectively. As the DLS result shows the diameter of the nanodiscs were very close to the expected molecular size.

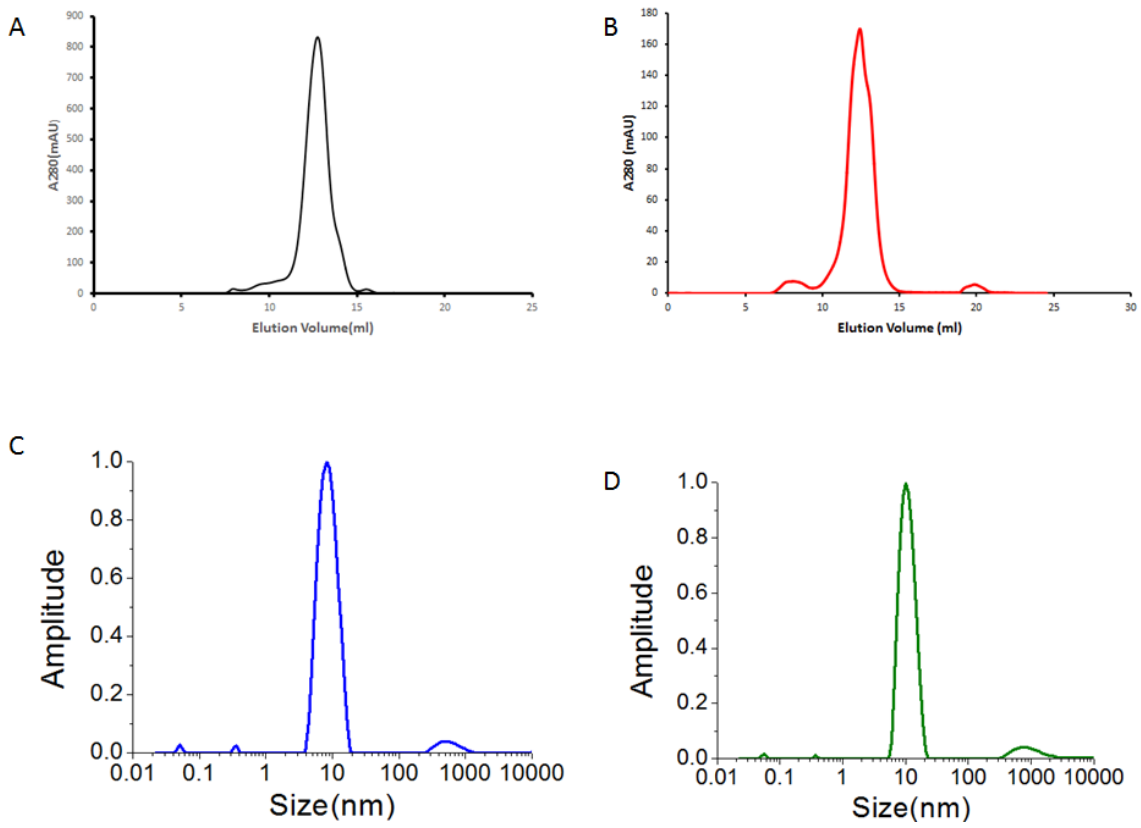


Figure 11: Purification and size distribution of different nanodiscs (A) Purification of MSP1D1 and (B) Purification of MSP1E3D1 (C) size distribution of MSP1D1 and (D) size distribution of MSP1E3D1 by dynamic light scattering.

4.3.Expression and productivity of membrane proteins in the PURE system

To understand the functional and structural roles of a repertoire of GPCRs, we systematically examined their expression and productivity by the PURE system. A PURE system though it lacks cellular membrane, its open nature makes it amenable to incorporate lipids, detergents, micelles and other important factors as per the objective. In this particular experiment, we supplemented the PURE system with lipid bilayers (nanodiscs and GUVs), micelles and also detergents and evaluate the expression of the G-protein coupled receptors such as CX_3CR1 and CCR5 which are characterized by their seven transmembrane helices. As GPCRs are associated with cellular functions and physiological processes, the study of these class of membrane proteins plays a crucial role. To this end, we also screened mouse and human GPCRs by the PURE system and we selected GPCRs based on their physiological role and their productivity as well as solubility for further optimization and study.

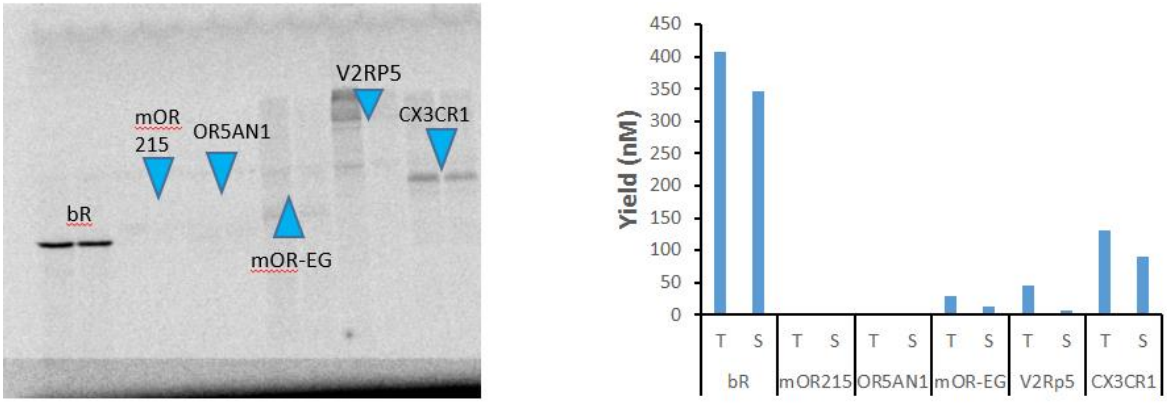
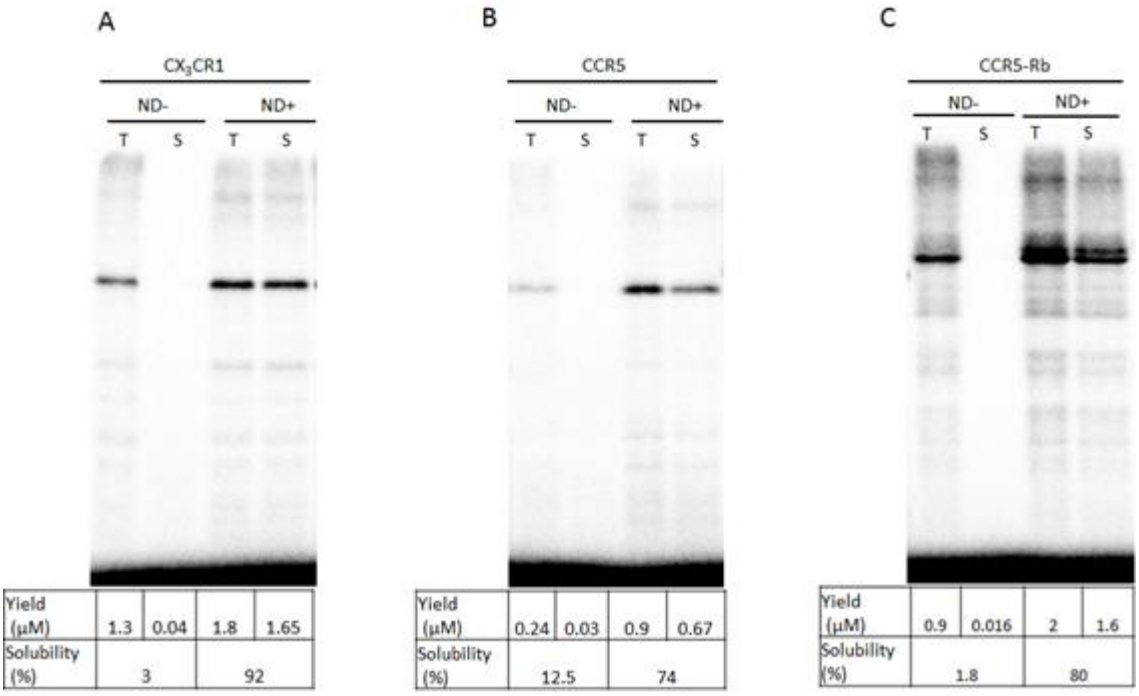


Figure 12: Screening of GPCRs by the PURE system. Human and mouse GPCRs were synthesized in the PURE system

Among these class of GPCRs, the chemokine receptors were expressed to a level of 100 nM with 70-80% solubility. By following various strategies such as optimizing the codon sequence, and improving the PURE version has increased the productivity nearly 10 times. Therefore, we also expressed CCR5 chemokine receptors by the improved condition.

To further understand the importance of nanodiscs for solubilization of GPCRs, we expressed selected chemokine GPCRs in the PURE system containing ^{35}S Methionine in the presence and absence of nanodiscs. In the absence of nanodiscs, GPCRs were aggregated and appeared in the soluble fraction with insignificant amount during centrifugation assay. In both cases, the total fraction and soluble fractions were run on 15% SDS-PAGE and the productivity were quantified by autoradiography. A productivity of 0.05 mg, 0.04 mg and 0.1 mg per ml of PURE reaction obtained for CX_3CR1, CCR5 and CCR5 respectively.



Yield (μM)	1.3	0.04	1.8	1.65
Solubility (%)	3		92	

Yield (μM)	0.24	0.03	0.9	0.67
Solubility (%)	12.5		74	

Yield (μM)	0.9	0.016	2	1.6
Solubility (%)	1.8		80	

Figure 13: Cell-free expression and respective solubility of GPCRs. The GPCRs were expressed in the PURE system supplemented with [^{35}S]-Methionine and in the presence or absence of POPC nanodisc (ND+ or ND-, respectively), and the solubility was subsequently estimated as in the case of CX_3CR1 **(A),** CCR5 **(B)** and CCR5-Rb **(C)**. The soluble fraction was quantified by dividing the supernatant (S) by the total (T) amount of synthesized protein.

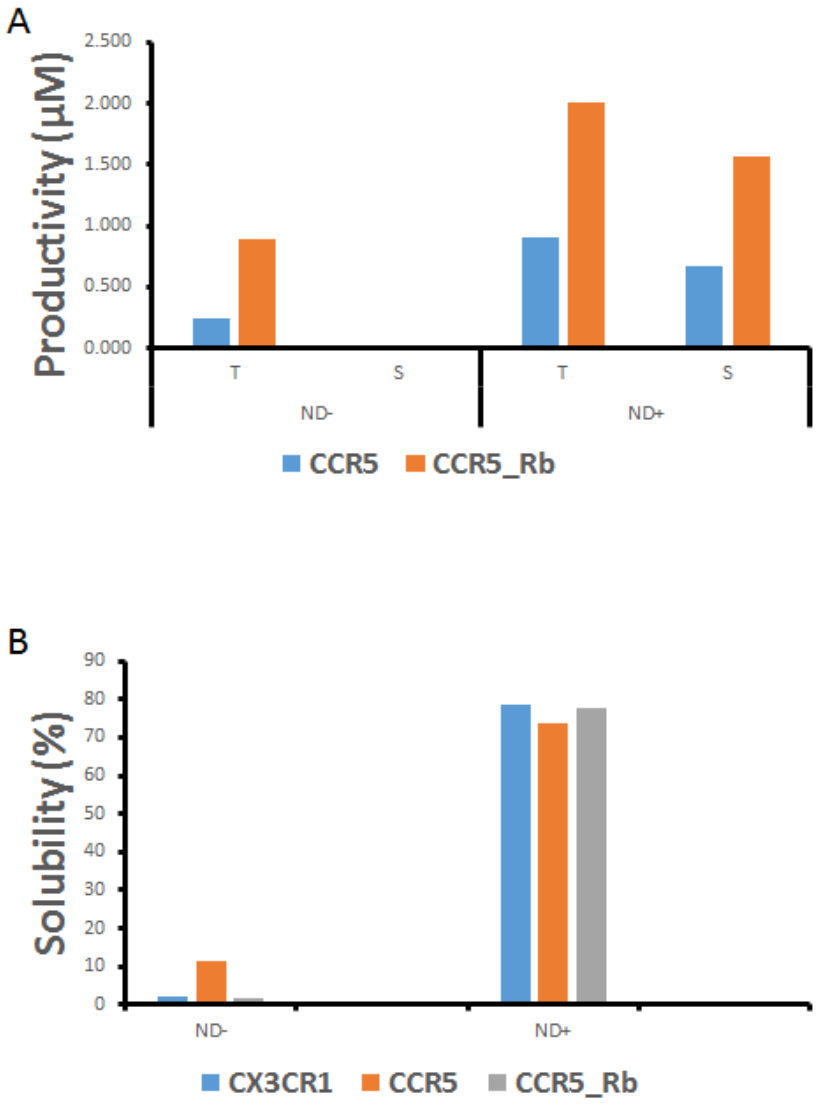


Figure 14: Productivity and solubility of PURE expressed GPCRs under different conditions. (A) Comparison of productivity of CCR5 (blue) and CCR5_Rb (orange) in the absence of nanodiscs (ND-) and in the presence of nanodiscs (ND+). (B) Solubility of CX3CR1 (blue), CCR5 (orange) and CCR5_Rb (grey) in the abscence of nanodisc (ND-) and presence of nanodiscs (ND+).

4.4.Effect of Rubredoxin on expression of CCR5 and solubility

A 54 amino acid residue, rubredoxin (Rb), inserted into the ICL3 of CCR5 was used during the determination of the crystal structure to enhance stability. So, we analyzed the effect oon a cell-free synthesis of CCR5 for productivity and solubility. The CCR5 containing the insert increased the productivity twice (2 µM) as compared to 0.2 µM without insert as a total fraction. The soluble fraction at the same time increased twice from 0.6 µM (CCR5) to 1.5 µM (CCR5_Rb) in the presence of nanodiscs (ND+). In the presence of nanodiscs, the effect of the insert on solubility has been investigated and there is no any significant difference although the overall soluble fraction increased slightly.

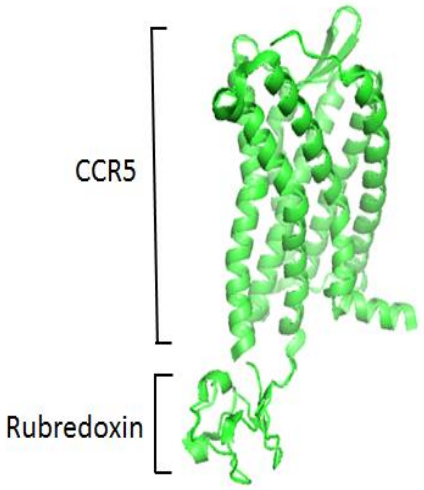
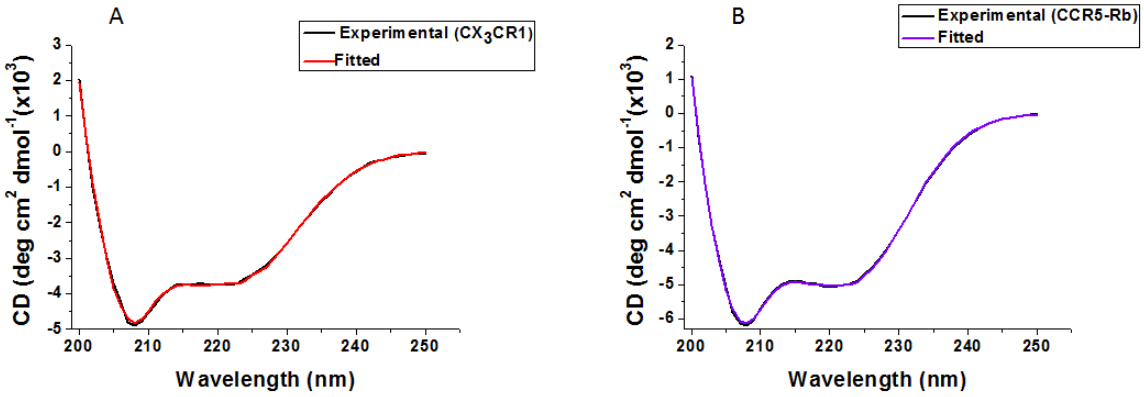


Figure 15: Crystal structure of CCR5 GPCR. For stabilization, a 54 amino acid residue rubredoxin Rb) was inserted at ICL3

4.5.Secondary structure of CX3CR1 and CCR5 in Micelle

The secondary structures are a measure of the α-helix content of membrane proteins. Thus, PURE synthesized receptors, CX_3CR1 and CCR5 were estimated by circular dichroism. As the membrane scaffold protein in nanodisc is alpha helical and interferes with CD measurement of receptors, a detergent or micelle is appropriate for CD analysis. Hence, we synthesized both CX_3CR1 and CCR5 by the PURE system containing a micelle and purified receptors in CD buffer were used for CD analysis. The CD spectra showed a minima wavelength at 208 nm and 222 nm that is a characteristic feature of α-helix proteins (Figure 3A and B). The analysis by BeStSel software [32] showed that a 21.5% and 49.5% α-helix content was obtained for CX_3CR1 and CCR5-Rb receptor proteins respectively.



CX_3CR1 secondary structure (%)	
Helix	40
Beta	9.2
Turn	13.2
Others	37.7

CCR5-Rb secondary structure (%)	
Helix	49.5
Beta	5.3
Turn	13.2
Others	32.1

Figure 16: CD measurement of cell-free synthesized GPCRs.Secondary structures of PURE-synthesized CX_3CR1 (**A**) and CCR5-Rb (**B**) in the presence of a micelle (25 mg/mL brain polar lipid and 75 mg/mL digitonin) were analyzed. For CD measurement, the detergent was exchanged with a buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% DDM and 0.002% CHS during Ni^{2+} column chromatography.

4.6.Interaction of CX3CR1 with CX3CL1 chemokine domain in nanodisc

The binding of chemokine ligands to chemokine receptors activate a cascade of physiological processes and intracellular signaling. The functions of both inflammatory and chemoattractant chemokines are mediated by their interaction with the receptor. Therefore, a malfunction in the chemokine ligand or receptor affects the interaction and results in pathophysiological disease such as inflammation and neurodegenerative disease which includes allergic asthma, atherosclerosis, Alzheimer's and parkinson's disease. To delineate the specific residues involved in ligand binding and intracellular signaling, a site-directed mutagenesis both in the chemokine ligand and the receptor was studied described in [33, 34].

In this study, we employed a label-free surface plasmon resonance method to determine the affinity and kinetics of ligand-receptor interactions by immobilizing PURE expressed receptors in nanodisc (Figure 4A) or directly the ligand (Figure 4B) without prior purification. As SPR requires less amount of sample and due to its high sensitivity. After equilibration of CM5 sensor chips with a running buffer, anti his antibody was immobilized to a level of ~900 RU and ~1500 RU of CX_3CR1-nanodisc complex was immobilized for interaction analysis.

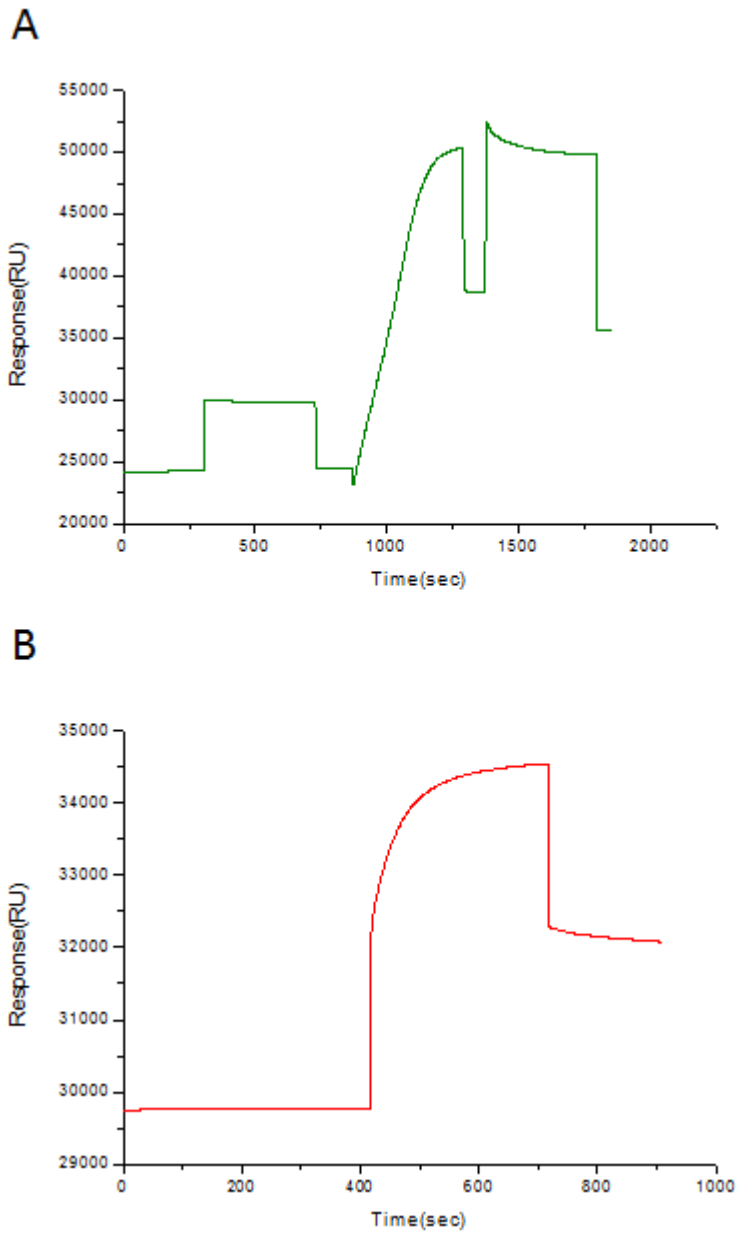


Figure 17: Preparation of CM5 sensor chips for ligand binding assay. Immobilization of (A) antihis antibody and (B) CX3CR1-nanodisc complex.

A single cycle kinetics was carried out to determine the kinetics of the interaction because this approach doesn't require optimization of regeneration conditions. In this particular experiment, we injected the analyte (the chemical domain of CX_3CR1) at a concentrations of 2.5 nM, 5 nM, 10 nM, 40 nM and 80 nM and monitored the binding. A measured binding affinity of 27.8 nM in nanodisc (Figure 4C) and 39.4 nM in a micelle (Figure 4D) were obtained.

4.7. Micelles for interaction analysis of CX3CR1 and the chemokine domain of CX3CL1

Most chemokine receptors are expressed in the brain and the use of brain polar lipid extracts play a critical role to examine the lipid effect in the activity of chemokine receptors. In this study, we only check activity of CX_3CR1 in a micelle. As a first step, the brain polar lipid was dissolved by a detergent, digitonin, added to the PURE system containing sfGFP fused to CX_3CR1 at the C-terminus and incubated at 37 ^{0}C for 6 hrs. After synthesis, it was subjected to Ni-IMAC chromatography and used as analyte for the interaction assay at a concentration of 2.5 nM, 5 nM, 10 nM, 40 nM and 80 nM.

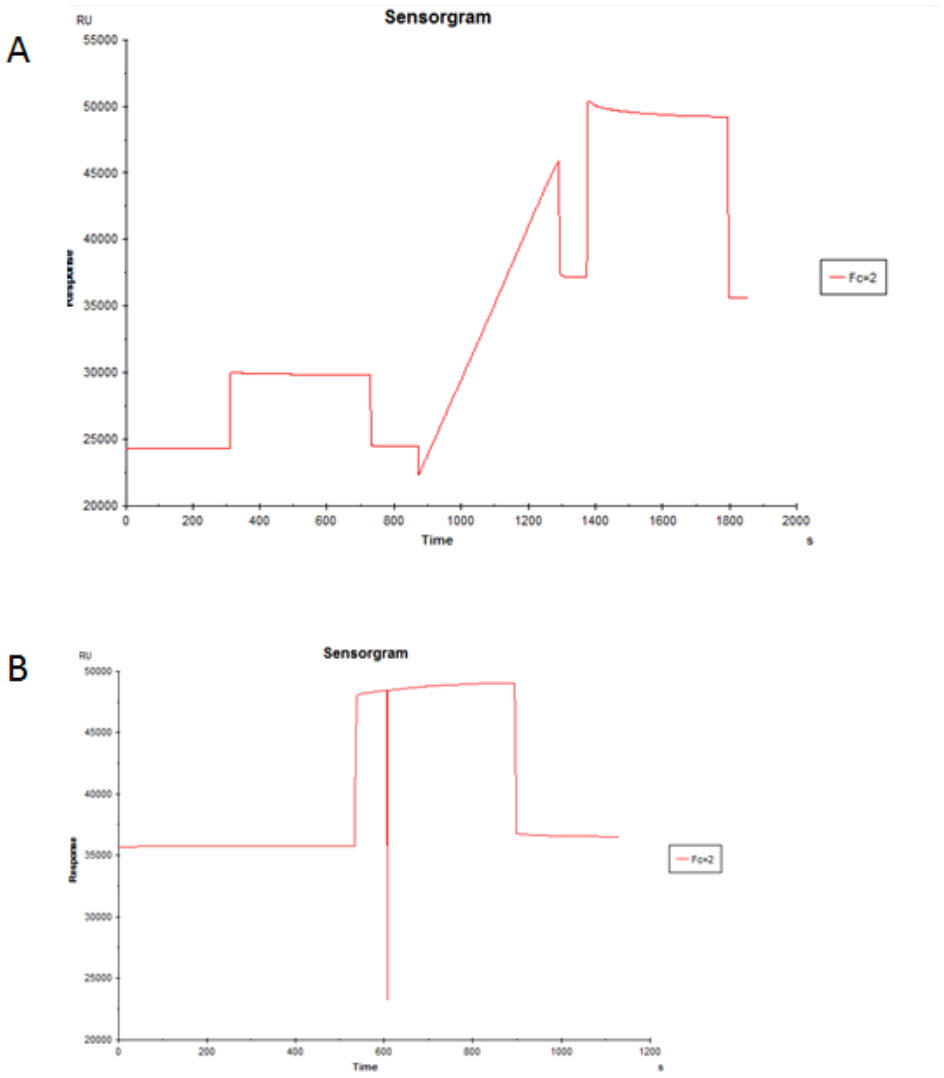


Figure 18: Preparation of CM5 sensor chips for ligand binding assay. Immobilization of (A) antistrp-tag II antibody and (B) the chemokine domain of CX_3CL1.

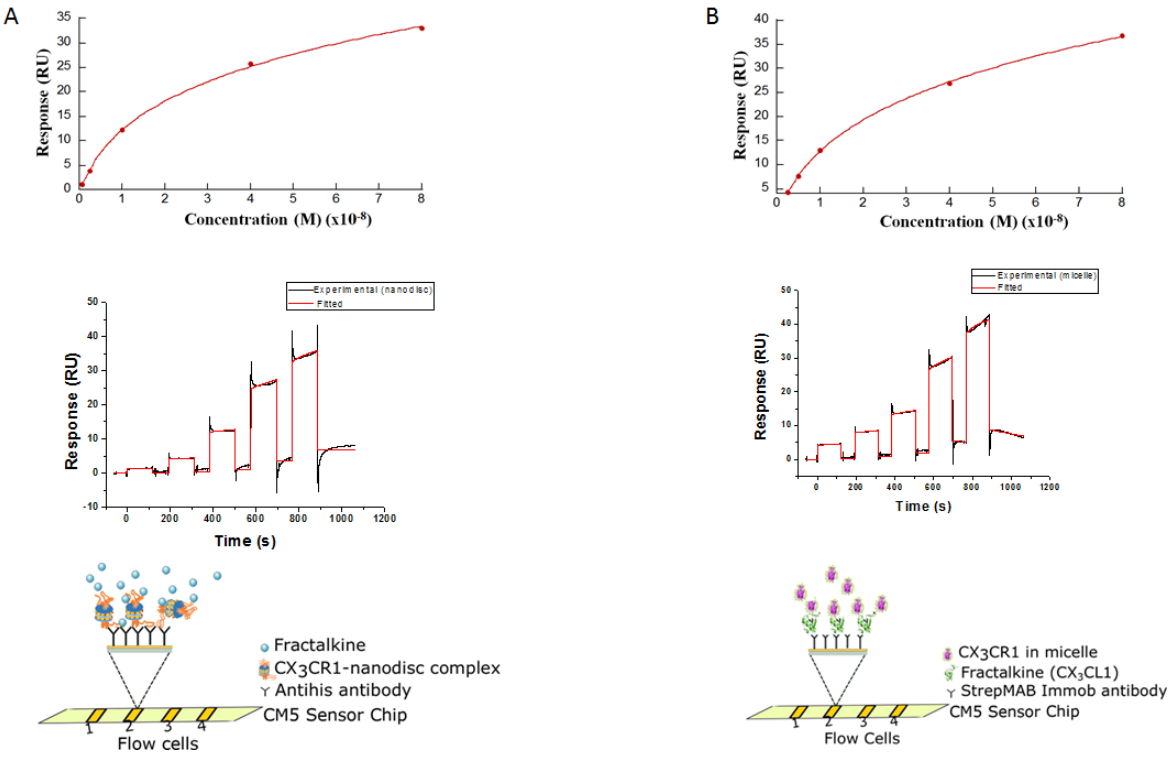


Figure 19: Receptor (CX3CR1)-ligand (CX3CL1) interaction in different environments. Schematic representation of receptor-ligand interaction in nanodisc (**A**) and micelle (**B**). In both (**A**, **B**), the upper, middle and lower figures correspond to the binding affinity constant, fitted kinetics and schematic representations of the immobilization strategy respectively. In all cases, the analytes at 2.5 nM, 5 nM, 10 nM, 40 nM and 80 nM concentrations were injected to determine the binding affinity constant using single cycle kinetics. For the nanodisc system, CX_3CL1 protein is denoted as the analyte and for the micelle system, His-tagged CX_3CR1 is denoted as the analyte.

4.8.Localization of G-protein coupled receptors (GPCRs)

The incorporation of target mebrane proteins into the lipid bilayer during a co-translational integration is a major question raised by many researchers. Thus, we investigated the localization of the membrane proteins by expressing the receptor CX_3CR1 fused with sfGFP at the C-terminus and probed by confocal microscopy. As a control, we used sfGFP alone. Hence, we confirmed the localization and integration of CX_3CR1 fused with sfGFP (Figure 6E) but not sfGFP (Figure 6F) in giant unilamelar vesicle. Unlike sfGFP, CX_3CR1 fused with sfGFP, localizes on the membrane

For transmission electron microscopy based image analysis, a homogeneous sample is critical. As a result, we purified the nanodisc-CX_3CR1 complex by size exclusion chromatography (Figure 5A). Both empty nanodiscs and CX_3CR1-nanodisc complexes appeared as a homogenous size distribution. The elution fractions appeared as a discrete band on a 15% SDS-PAGE with little contaminants from the PURE components (Figure 5B). The size distribution of empty nanodiscs (Figure 6A) and CX_3CR1-nanodisc complex (Figure 6B) were examined by transmission electron microscopy. Furthermore, TEM images of empty nanodiscs were analyzed by ImageJ (Figure 6C) and DLS (Figure 6D) and the size distribution was homogeneous with a diameter of 12-13nm.

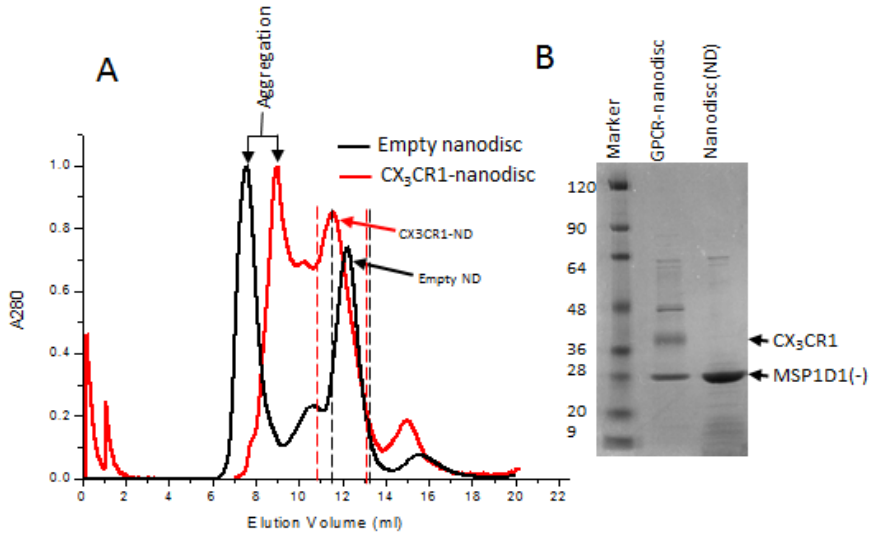


Figure 20: Purification of CX3CR1-nanodisc complex. **(A)** Elution pattern of the CX_3CR1-nanodisc complex (red) and empty nanodisc (black) as observed during the size exclusion chromatography. The peak was normalized to the elution profile of empty nanodisc. **(B)** SDS-PAGE analysis of the elution fraction stained by Coomassie Brilliant Blue (CBB).

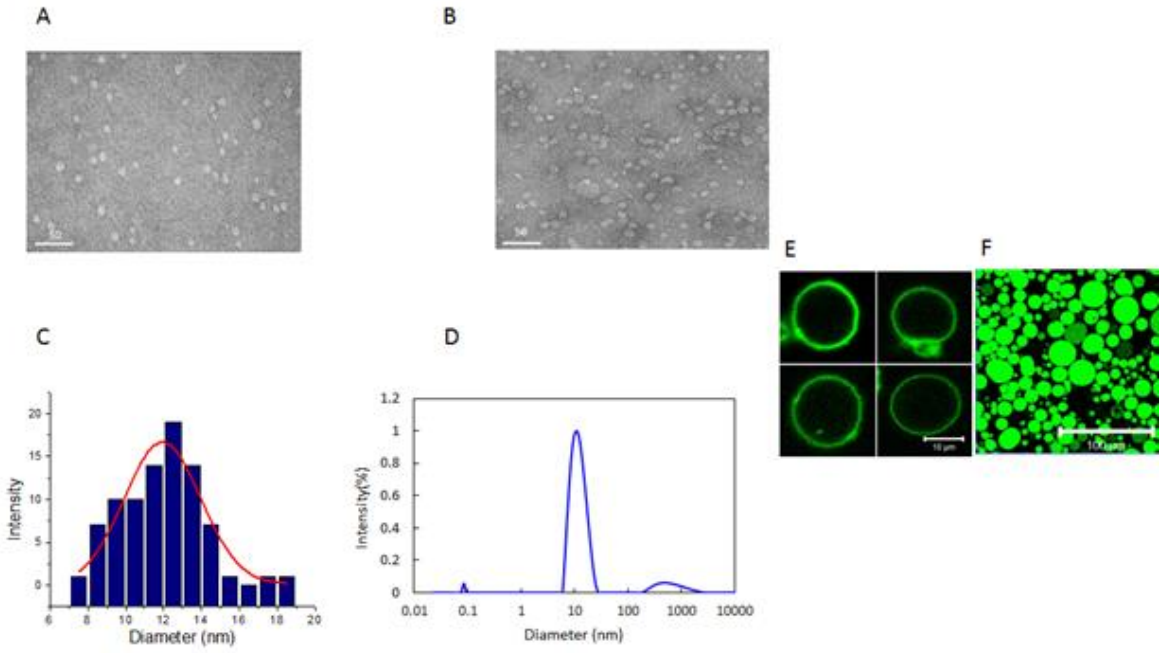


Figure 21: Spontaneous membrane localization of cell-free synthesized receptor protein.Transmission electron microscopy micrographs of empty nanodisc **(A)** and CX_3CR1-containing nanodiscs **(B)** at 30,000x magnification. The size distribution of empty nanodiscs as measured directly by imageJ software **(C)** and dynamic light scattering **(D)**. **(E)** Spontaneous membrane integration of CX_3CR1-sfGFP synthesized inside GUV. **(F)** sfGFP synthesized inside GUV as a control.

5. Discussion

5.1. Productivity and solubility of chemokine GPCRs in a cell free system

Recently, various functional membrane proteins were synthesized by cell-free systems containing detergents, chaperones, micelles, liposomes and nanodiscs. These include C-C Chemokine receptor type 5 (CCR5) [32], claudin-4 [31], secYEG [33] and human endothelin B receptor (ETB) [34]. The nanodisc technology has been used for the reconstitution of various membrane proteins such as a G-protein coupled receptor, CCR5 [35], an ion channel TRPV1 [36] and a transporter, MsbA [37].

Cell-free translation systems are used to produce target proteins within a few hours and they are expected to supersede the cell-based expression for functional analysis of membrane proteins due to the advantage in expression and purification processes. In synthetic biology, cell-free systems are indispensable to construct artificial cells capable of mimicking the cellular environment for the investigation of difficult-to-express membrane proteins such as the G-protein coupled receptors [38,39]. Thus, producing GPCRs in a cell-free system is inherently challenging due to their aggregation properties and demands in the optimization of the system to obtain active proteins. It was demonstrated by Chi et al. [32] that supplementing the cell-free system with chaperones yields a functional CCR5 with the expected binding affinity. Chemokine receptors and their ligands play a crucial role in the immune system and implicated in various pathophysiological disease [40,41]. However, functional studies of GPCRs in vitro was mainly hampered by precipitation. Thus, translation of CX_3CR1 and CCR5 chemokine GPCRs in a cell-free system without modification of the system through the addition of lipid bilayers and micelles leads to aggregation and low productivity (Figure 2). The stability and productivity of GPCRs can further be improved by the insertion of a thermostabilizing fragment such as an iron-sulfur redox protein, rubredoxin, at the third intracellular loop of target GPCRs [42]. In our system, insertion of rubredoxin (Rb) to CCR5 improved the productivity in the presence of nanodisc (ND+) by twofold and by fourfold in the absence of nanodisc (ND-) as compared to the yield of CCR5. Whereas the solubility showed no significant improvement despite rubredoxin insertion.

5.2.Secondary structure analysis of CX_3CR1 and CCR5 in micelles

The heptahelical transmembrane of GPCRs is characterized by their α-helix content. Wiktor et al. [43,44] investigated the secondary structure of CCR5 expressed in *E. coli* and confirmed the presence of a minima at around 208 nm and 222 nm which is a typical characteristic of α-helix proteins. As the scaffold protein in nanodiscs contains α-helical structure which interferes with CD measurements, CX_3CR1 and CCR5 chemokine receptors were expressed by the PURE system supplemented with detergents and micelles. Micelles composed of a brain polar lipid and digitonin detergent were appropriate as most chemokine receptors are expressed in the brain [45,46]. Hence, the brain polar lipid containing a mixture of lipids is expected to provide enhanced stability and activity of CX_3CR1 and CCR5 chemokine receptors. Therefore, we examined the secondary structure of CX_3CR1 and CCR5-Rb synthesized by the PURE system in the presence of a micelle and obtained a typical characteristic of folded α-helix identical to the in vivo expressed GPCRs [41]. Though structural analysis of cell-free synthesized CX_3CR1 is necessary to confirm proper folding, the secondary structure indicates the presence of proper folded CX_3CR1 and CCR5-Rb synthesized by the PURE system (Figure 3) [43,44]. Nonetheless, the fraction of properly folded receptors in the PURE system is unknown and not yet determined from the secondary structure measurement. Furthermore, NMR analysis of cell-free expressed chemokine GPCRs through 13CH3 methionine will be a substantial approach to better understand the conformational changes of the receptor and will strengthen the secondary structural analysis as it was reported for other classes of GPCRs (36).

5.3.Functional analysis of CX_3CR1 through ligand binding

The interaction of chemokine receptors with their respective ligands determine the functionality of synthesized receptors. Surface plasmon resonance is a label-free assay method used to study ligand-receptor protein interactions and capable of measuring the interactions with high sensitivity and robustness [47]. Shepherd et al. [48] and Rues et al. [49] applied SPR to determine the binding affinity constant of CCR5 in detergent and β1AR reconstituted into nanodiscs. In addition, Shinoda et al. [31] determined the binding affinity constant for cell-free synthesized claudin-4 in a micelle by SPR. Taking advantage of the

manipulation of the PURE system reaction condition, we synthesized CX_3CR1 in the presence of nanodiscs and micelles and measured ligand-receptor interactions.

CX_3CR1 synthesized by the PURE system was directly immobilized on a sensor chip pre-immobilized with anti-His antibody and a binding affinity comparative to the affinity determined by radioligand binding of in vivo expressed CX_3CR1 protein was achieved [50]. Nanodiscs enhanced the stability of membrane proteins by lowering the dissociation rate constant and minimizing the disparity in binding affinity constants compared to other methods [51]. Similarly, we succeeded in the synthesis of a chemokine ligand, CX_3CL1, by the PURE system and immobilized directly on a sensor chip pre-immobilized with StrepMAB-Immo antibody. CX_3CR1 synthesized by the PURE system containing a micelle, interacted with immobilized ligand and a binding affinity was determined. The binding affinity constants for both systems are outside the range of previously reported values of 1–4 nM by radioligand binding [35]. The possible reason for the discrepancy in binding affinity constant can be explained by the fact that different assay systems or experimental conditions result in a different calculated affinity constant.

6. Conclusion and future perspective

The advent of cell-free systems and the nanodisc technology are future promises in the study of membrane proteins. The synergy between cell-free system and nanodisc minimizes the time of target protein production, avoids detergent contact which is detrimental for the activity of some membrane proteins during the purification process, allows to control the lipid environments and other factors and avoids toxicity effect, which arises during cell-based expression, and become amenable to produce toxic products.

As most GPCRs orphan GPCRs lacking an endogenous ligand, the use of a cell-free system in combination with nanodiscs allows us to explore specific ligands capable of activating the receptor from a pool of peptide library. This method is also applicable for high throughput screening of drugs.

Both co-translational integration and post translational reconstitution of membrane proteins at large and GPCRs in particular are key for the study of single molecule and ligand binding assay which requires stable immobilization. The nanodiscs provide a near native environment for membrane proteins and increase the overall molecular mass which is a key requirement for cryo-EM based structural analysis.

Therefore, our system has a great potential for investigating the functions of membrane proteins including orphan GPCRs. Furthermore, our system can easily be adapted to study oligomerization of GPCRs and other membrane proteins.

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