Master's Thesis of Chemical and Biological Engineering

Development of GPCR-embedded Nanodisc Platform for Small-molecule Drug Screening

저분자 약물 스크리닝에 적용 가능한 GPCR 포함된 나노디스크 플랫폼 개발

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Abstract

G protein G protein-coupled receptors (GPCRs) constitute the largest receptor family in the human genome, regulating many physiological and pathological processes. Naturally, 34% of all marketed drugs target GPCRs. The 5-HT_7 receptor (5-HT_7R), the most recently identified member of the 5-HT receptors, is one of the most highly targeted GPCRs in the market with over 20 distinct approved agents. The 5-HT_7 receptor regulates many different functions such as circadian rhythm, depression, learning, memory, nociception, etc. The parathyroid hormone receptor (PTHR) helps regulate bone, kidney, and intestine functions and is targeted for conditions such as osteoporosis.

However, the receptors are still yet to be fully exploited for therapeutic purposes, one of the reasons being a lack of sensitive tools to properly identify possible ligands. By employing the nanodisc platform, which mimics the native structure of the receptor in a cell, the 5-HT_7 and PTH receptor can be reconstituted to be used in selective and sensitive drug screening platform for the detection of 5-HT_7 and PTH receptor ligands. We expressed the 5-HT_7 and PTH receptor from *E. coli* and reconstituted it in nanodiscs consisting of receptor, membrane scaffold protein, and phospholipid bilayer to mimic the membrane environment. Together with the fluorescence polarization assay, the GPCR receptor nanodisc represents an attractive item for use in drug screening platforms.

Keyword : GPCR, 5-HT_7R, PTHR, Nanodisc

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Table of Contents

List of Figures

1. Introduction

1.1 5-HT_7 receptor and parathyroid hormone receptor

G protein-coupled receptors (GPCRs) are transmembrane proteins with seven α-helical domains. They are involved in various physiological processes, such as hormonal signaling, neuronal transmission, sensory signaling, and cell signal transduction [1]. GPCRs account for more than 34% of all marketed drug targets. Therefore, the reconstitution of GPCRs is critical for any receptor-related research.

The 5-HT_7 receptor (5-HT_7R), the most recently identified member of the 5-HT receptors, is a family A GPCR and one of the most highly targeted GPCRs in the market with over 20 distinct approved agents. The 5-HT_7 receptor regulates many different functions such as circadian rhythm, depression, learning, memory, and nociception [2]. Its ligands can affect dopamine, GABA, and glutamate neurotransmission [3]. Currently, the FDA-approved drugs that target 5-HT_7R are used for depression or memory loss.

The parathyroid hormone receptor (PTHR) is a family B GPCR that regulates calcium and phosphate homeostasis. PTHR affects bone, kidney,

and intestinal functions by releasing or reducing calcium levels and activating vitamin D [4]. PTHR is currently a drug target for osteoporosis and hypoparathyroidism. Agonists such as parathyroid hormone-related protein help increase the bone mineral density, which, if too low, can induce osteoporosis. PTH is used to treat hypoparathyroidism, which induces cramping and twitching [5].

However, the receptor is still yet to be fully exploited for therapeutic purposes, one of the reasons being a lack of sensitive tools to properly identify possible ligands. By employing the nanodisc platform, which mimics the native structure of the receptor in a cell, the 5-HT_7 and PTH receptors can be reconstituted to be used in selective and sensitive drug screening platforms for the detection of 5-HT_7 and PTH receptor ligands. We expressed the hormone receptors from *E. coli* and reconstituted it in nanodiscs consisting of 5-HT_7 or PTH receptor, membrane scaffold protein, and phospholipid bilayer to mimic the membrane environment. Altogether, the 5-HT_7 and PTH receptor nanodisc represents an attractive item for use in drug screening platforms.

1.2 Expression and purification of G protein-coupled receptor (GPCR)

1.2.1 Expression of GPCR

GPCRs were expressed in *E. coli* systems. Mammalian and insect cells are traditionally the most often used systems for synthesis and post-translational processing of eukaryotic receptors, but they are too costly to attain the amount of necessary proteins for this research. In yeast cells, the GPCRs are often folded incorrectly [6]. Bacteria such as *E. coli* does not require costly media or equipment. The *E. coli* system also has the advantage of being easily scaled. Based on the above information, *E. coli* cells represent a promising system for expression of GPCRs.

1.2.2 Purification of GPCR

GPCRs have strong tendencies to be produced as inclusion bodies in *E. coli* [7]. Therefore, in order to study GPCRs for protein-based research, they first need to be solubilized. The solubilization of GPCRs, specifically hormone receptors, requires selecting the right detergent. Triton X-100, DDM, Tween, cholate, and urea are famous examples of detergents used in this process [8]. However, SDS, an anionic detergent, is often the detergent of choice when solubilizing GPCRs and producing nanodiscs. After a dialysis process to rid of EDTA in the solubilized protein, the His-tagged

protein can be purified through nickel column chromatography via HisTrap column. Insoluble proteins have the advantage of higher purity over soluble proteins because the soluble fractions are already separated during the lysis process.

1.3 Functional reconstitution of GPCR

There are several methods to reconstitute GPCRs such as detergent micelles, lipid vesicles, and bicelles [9, 10, 11]. However, among these techniques, nanodiscs are considered the optimal platform for reconstitution. Regardless, the above techniques all share three common steps. Firstly, proteins need to be solubilized in detergents. Secondly, affinity purification is conducted to purify the membrane protein. Lastly, the detergent is removed by dialysis or adsorption techniques such as the application of Bio-Beads.

1.3.1 Nanodiscs

Nanodiscs consist of three parts: a receptor protein, a membrane scaffolding protein (MSP), and a lipid bilayer. The lipid bilayer provides a native like environment and the MSP wraps around the lipid structure,

functioning as a stabilizing belt for the nanodisc structure as seen in **Figure 1.1**.

The nanodisc platform possesses several key advantages over its other counterparts. First, the nanodisc structure is more compact than other reconstitution structures, with less free-floating lipids or detergents, since MSP functions as a belt holding the structure together [12]. Second, MSP can also be used in effectively labelling nanodiscs. Fluorescent amino acids can be incorporated into the MSP sequence prior to expression in *E. coli,* allowing for easy labeling [13]. Third, the nanodisc structure is table and monodisperse, offering advantages in structure determination of GPCRs, which is arguably its main application [14]. Nanodiscs are the platform of choice for structural studies of membrane proteins, often used in tandem with X-ray crystallography and electron microscopy.

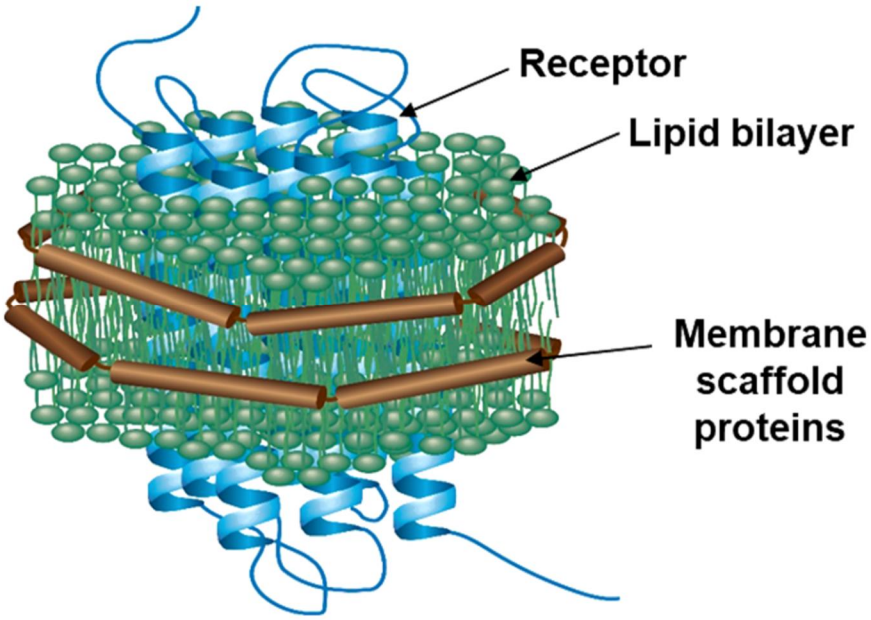


Figure 1.1 Structure of receptor-embedded nanodiscs

2. Materials and methods

2.1 Gene cloning

The 5-HT_7R gene was amplified by PCR with primer (5' ATG AAT TC ATG ATG GAC GTT AAC AGC 3', 5' ATT CTA GAT TAC AGC ACA AAC TCA GG 3') using pcDNA3.1 containing 5-HT_7R cDNA. The PCR product was cloned into the bacterial pET-DEST42 expression vector (Invitrogen) using the gateway cloning system of pENTR/D-TOPO (Invitrogen) and LR clonase (Invitrogen). The PTHR gene was amplified by PCR with primers (5' ATG AAT TCA TGG GGA CCG CCC G 3', 5' ATG CGG CCG CTC ACA TGA CTG TCT CCC A 3') using pcDNA3.1 containing PTHR cDNA. The PCR product was cloned into the bacterial pET-DEST42 expression vector (Invitrogen) using the gateway cloning system of pENTR/D-TOPO (Invitrogen) and LR clonase (Invitrogen).

2.2 Expression

2.2.1 Expression of GPCRs in *E. coli*

The Rosetta2 cells were transformed with the pET-DEST42 vector and cultured in LB medium (+100 μg/mL ampicillin) at 37°C until the OD_{600} value reached 0.5~0.6. GPCR expression was induced by the introduction of 1 mM IPTG and the cells were induced for 4 h. Afterwards, the cells were

centrifuged (8000g, 20 min, 4°C) with the supernatant discarded. The harvested cell pellets were then resuspended in PBS, 2 mM EDTA. The cells were lysed by sonication with a pulse and duration setting of 5s on/off, 5 min. After further centrifugation (8000g, 20 min, 25°C), the pellet of the sonicated sample was collected as the insoluble fraction containing the desired GPCR.

2.2.1 Expression of membrane scaffold protein 1E3D1 in *E. coli*

Rosetta2 cells were transformed with the pET-28a vector and cultured in LB medium (+50 μg/mL kanamycin) at 37°C until the OD_{600} value reached 0.5~0.6. MSP expression was induced by the introduction of 1 mM IPTG and the cells were induced for 4 h. After 4h, the cells were centrifuged (8000g, 20 min, 4°C) and the supernatant collected as it contained the desired soluble fraction containing MSPs.

2. Purification

2.3.1 Purification of GPCRs in *E. coli*

The insoluble pellets of receptor proteins after sonication were solubilized in solubilization buffer (0.1 M Tris-HCl, 20 mM sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 1 mM EDTA, pH 7.4) at 25°C. The solubilized proteins were dialyzed against 0.1 M sodium phosphate (pH 8.0) containing 10 mM SDS using a 10K MWCO dialysis cassette (Thermo Scientific) and filtered with a 0.45 μm bottle top filter (Thermo Scientific) before applying the protein sample to a HisTrap HP column (Cytiva) equilibrated in the binding buffer (0.1 M sodium phosphate buffer, 10 mM SDS, pH 8.0). The protein was washed through a pH gradient from pH 8.0 to 7.0 and eluted with a pH 6.0 buffer. Afterwards, the eluted protein was desalted into the HEPES buffer II (20 mM HEPES- NaOH, 100 mM NaCl, 25 mM cholate, pH 8.0) by HiTrap Desalting column (Cytiva). The purified GPCR was analyzed by SDS-PAGE and western blot analysis.

2.3.2 Purification of MSP in *E. coli*

The *E. coli* cells from bacterial culture were harvested by centrifugation at 8000g for 20 min at 4°C. The protein was then solubilized in the binding buffer (20mM Tris-HCl, 0.5M NaCl, 20mM imidazole, pH 8.0) and filtered by a 0.45 μm bottle top filter before binding to the HisTrap HP column (Cytiva) using fast protein liquid chromatography (FPLC) (GE Healthcare).

MSP was washed with washing buffer (20 mM Tris-HCl, 50 mM imidazole and 0.5 M NaCl, pH 8.0) by increasing the imidazole concentration. The target protein was eluted with elution buffer (20 mM Tris-HCl, 350 mM imidazole and 0.5 M NaCl, pH 8.0). The purified MSP1E3D1 was desalted into the HEPES buffer II using the HiTrap HP desalting column (Cytiva). Afterwards, TEV protease was applied to the desalted MSP sample overnight in a 1:50 TEV to MSP molar ratio. The next day, the His-tag cleaved MSP was collected by taking the fraction that did not bind to the HisTrap HP column.

2.3.3 Western blot of GPCRs

Protein samples were inserted in 20 μL units and were analyzed by SDS-PAGE and western blot analysis. Western blot analysis was performed using anti-V5 epitope mouse antibody (Santa Cruz Biotechnology) as primary antibody. HRP-conjugated anti-rabbit antibody (Millipore) and HRP-conjugated antimouse antibody (Milipore) were used as the secondary antibodies.

2.4 Functional reconstitution

2.4.1 Functional reconstitution using nanodiscs

Palmitoyloleoylphosphatidylcholine (POPC) and 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were selected as the lipids of choice for nanodiscs because of their prominence in nanodisc assembly. The liquid phase lipids were placed in a round bottom flask, dried up by nitrogen gas, and placed in a vacuum overnight to remove extra chloroform. Afterwards, the lipids were dissolved in HEPES I buffer (20 mM HEPES-NaOH, 25mM cholate, 100 mM NaCl, pH 8.0).

Nanodisc assembly begins by mixing the receptor protein with MSP and incubating at the phase transition temperature of the relevant lipid. The phase transition temperature is 4°C for POPC and 25°C for DMPC. The receptor-MSP mixture is incubated at the specific temperature for 10 minutes before adding the lipid to the mixture. The mixed sample was incubated with a gentle stir for 2 h at 4°C or 25°C . The final mixture contained 5 μM receptor, 8 mM lipids, 25 mM detergents, and 33 μM MSP. Afterwards, Bio-Beads (Bio-Rad) were added to the mixture to remove any detergents (cholate, SDS) for 2h. Bio-Beads application can also be done overnight, but results may vary for different receptors. The mixture was injected through a 2mL loop into the size exclusion chromatography (SEC) (Superdex 200 Increase 10/300 GL, Cytiva) column to remove larger protein aggregates and smaller protein residues. The column was equilibrated with HEPES buffer II (20 mM HEPES-NaOH, 100 mM NaCl,

pH 8.0). The receptor-embedded nanodiscs were collected and stored at either 4°C or -80°C.

2.5 Characterization

2.5.1 Dynamic light scattering

The sizes of nanodiscs were analyzed using a dynamic light scattering spectrophotometer in NICEM, Seoul National University.

2.5.2 Western blot of nanodiscs

Western blot was performed on the nanodiscs to detect the successful incorporation of receptor proteins. The process was done in a manner identical to the process mentioned in section 2.3.1.

2.6 Functionality

2.6.1 Tryptophan fluorescence quenching assay

To prove the receptor-embedded nanodiscs' functionality, the tryptophan fluorescence quenching assay was performed by the spectrofluorometer with

excitation 290 nm and emission 340 nm conditions. Ligands of the receptors were applied to the nanodisc solution in different concentrations.

2.6.2 Fluorescence polarization assay

To use these GPCR nanodiscs as a drug screening platform, we had fluorescence-labeled tracers, ligands that we already know the binding affinities of, form a complex with the GPCR nanodisc. Fluorescence polarization assay measures the fluorescence polarization of excited fluorescent substances, with higher molecular weight fluorophores possessing higher fluorescence polarization.

The GPCR nanodisc/tracer complex has a higher molecular weight than the fluorescence-labeled tracer on its own, therefore having a higher fluorescence polarization. When receptor-specific drug candidates are applied to these complexes, the substitutions of labeled tracers by candidate drugs can be quantitatively measured by the decrease in fluorescence.

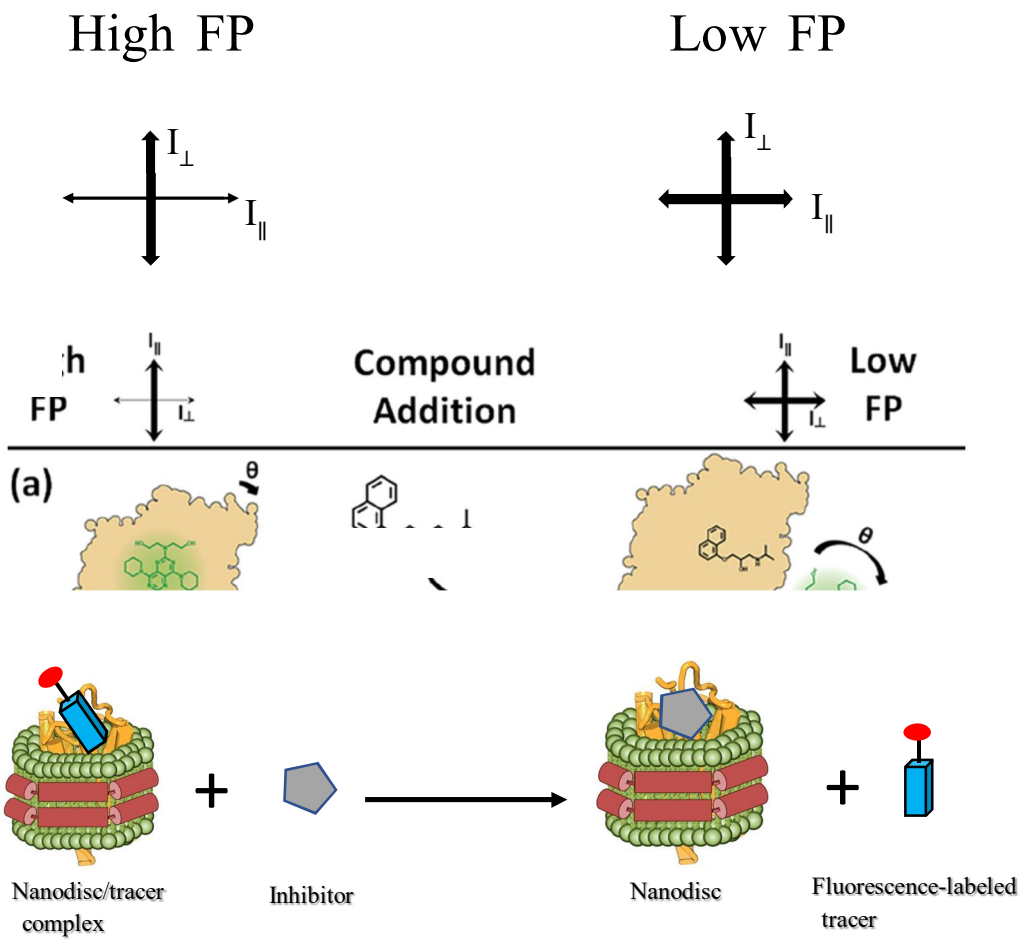
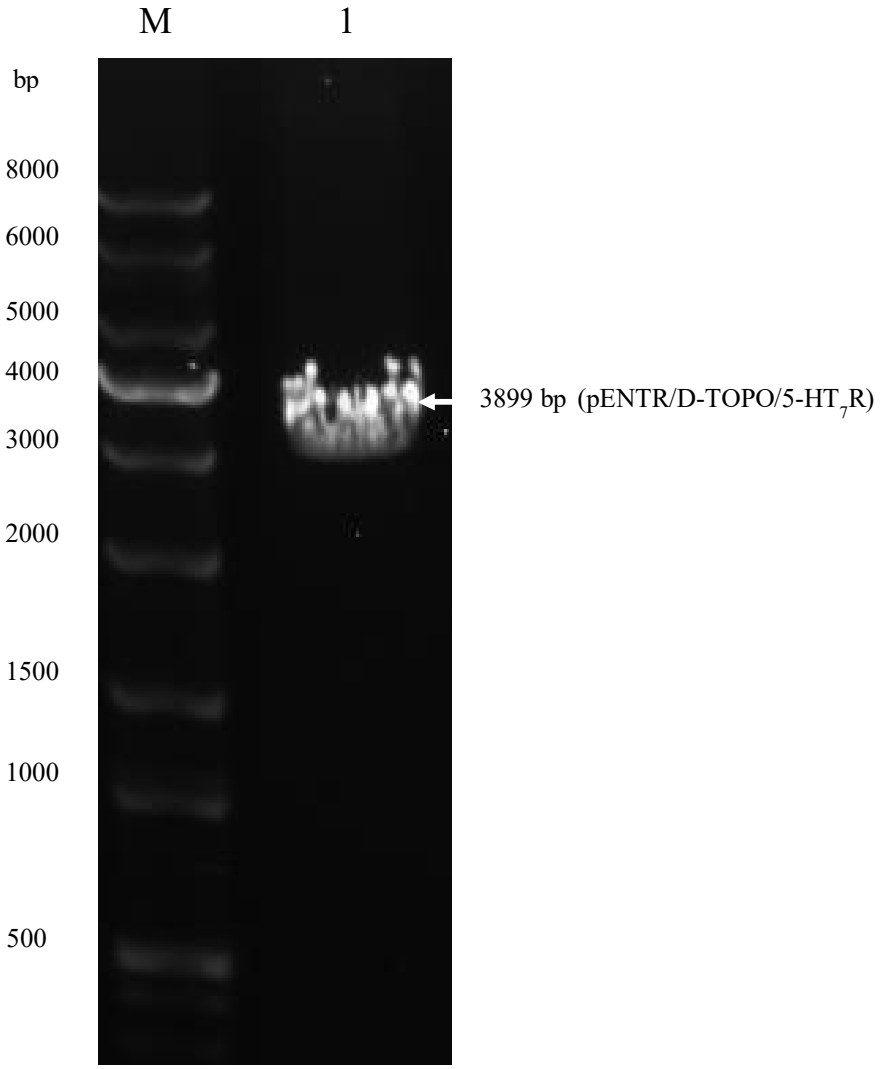


Figure 2.1 Mechanism of fluorescence-polarization assay as fluorescent-labeled tracer is substituted by a higher-affinity substance

3. Results and discussion

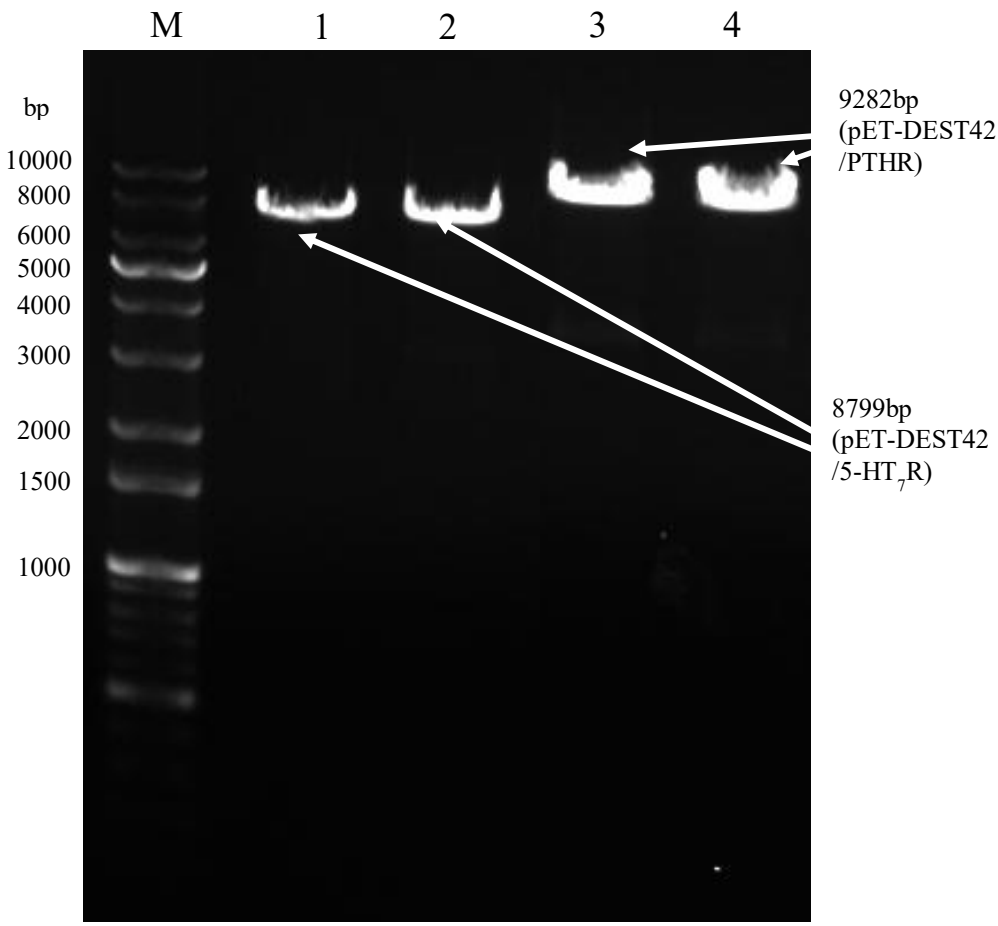
3.1 Gene cloning of GPCRs

After obtaining the PCR product from cDNA, the plasmids of 5-HT_7R and PTHR were first cloned into the pENTR/D-TOPO gateway cloning vector. The 5-HT_7R gene contains 1299 base pairs (bp) while the pENTR/D-TOPO vector contains 2600 bp, making the total bp 3899. The PTHR gene contains 1782 bp, so its total bp was 4382. After LR recombination cloning into the pET-DEST42 expression vector, which has a bp of 7500, the total bp of pET-DEST42/5-HT_7R and pET-DEST42/PTHR were 8799 and 9282 respectively. In **Figure 3.1** and **Figure 3.2**, the successful entry of GPCR plasmids into pENTR/D-TOPO and pET-DEST42 vectors are shown.



M: 1kb DNA marker
1: 5-HT_7R gene cloned into pENTR/D-TOPO vector

Figure 3.1 Gateway cloning of 5-HT_7R into pENTR/D-TOPO vector



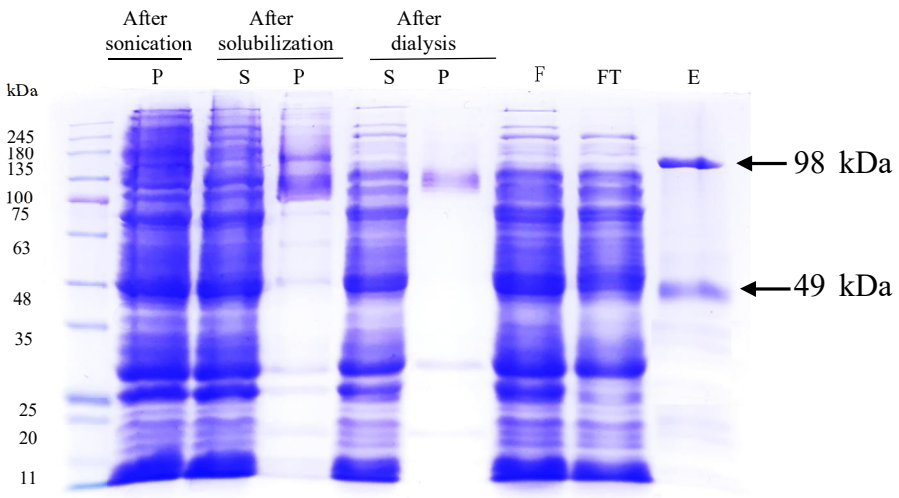
M: 1kb DNA marker
1, 2: 5-HT_7R gene cloned into pET-DEST42 vector
3, 4: PTHR gene cloned into pET-DEST42 vector

Figure 3.2 LR cloning of 5-HT_7R and PTHR into pET-DEST42 vector

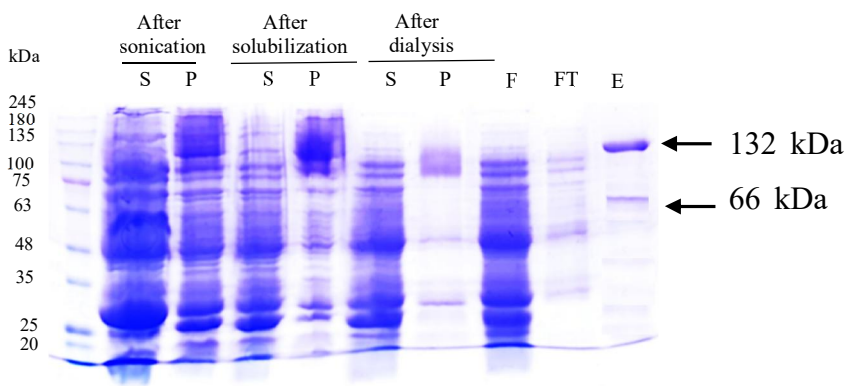
3.2 Expression and purification of GPCRs

While mammalian and insect systems are ideal for GPCR expression, *E. coli* systems can still successfully overexpress GPCRs by using certain vectors such as pDEST15 and pET-DEST42. Gel electrophoresis (SDS-PAGE) was performed for purified 5-HT_7R and PTHR expressed in *E. coli*. The 5-HT_7R and PTHR overexpressed by the *lacO* gene was sonicated, solubilized, and dialyzed in a 0.1 M sodium phosphate, 10mM SDS buffer, where they were purified via nickel column chromatography.

In Coomassie blue staining, 5-HT_7R was found at 49 kDa as a monomer and 98 kDa as a dimer, with two clear bands in the elution fraction. PTHR was found at 66 kDa as a monomer and 132 kDa as a dimer, with two clear bands in the elution fraction, shown in **Figure 3.3**. The two receptors were also analyzed in western blot via V5 as shown in **Figure 3.4** These results indicate that the two receptors were purified well.

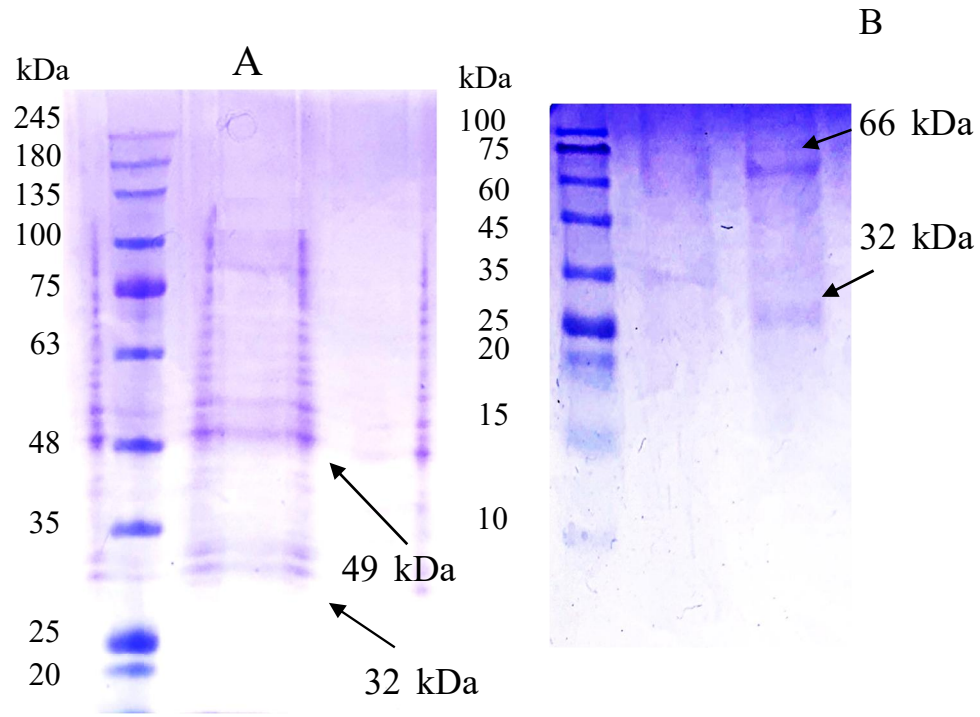


S: Supernatant after centrifugation
P: Pellet after centrifugation
F: Filtering
FT: Flowthrough
E: Eluent

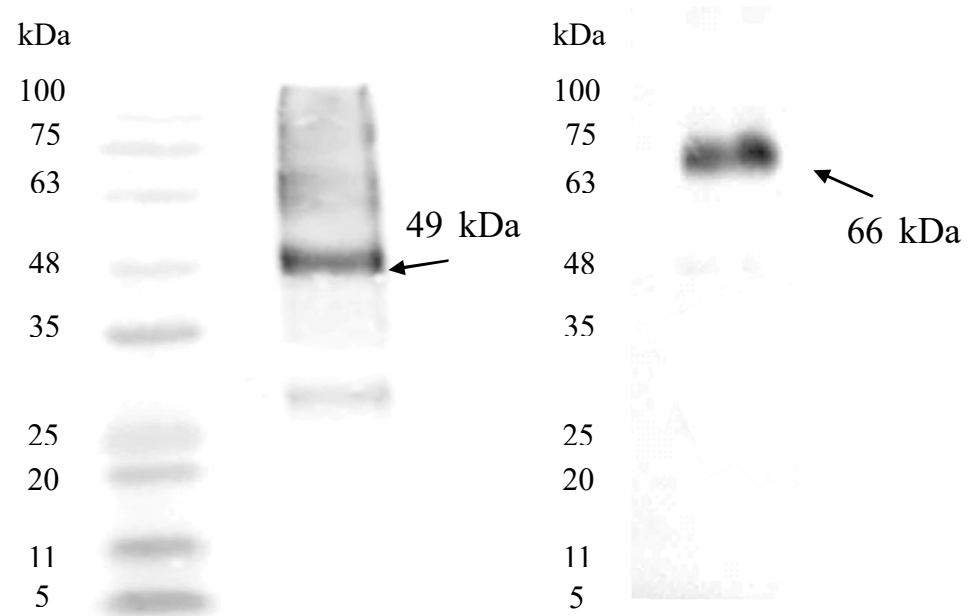


S: Supernatant after centrifugation
P: Pellet after centrifugation
F: Filtering
FT: Flowthrough
E: Eluent

Figure 3.3 Coomassie blue gel staining of 5-HT_7R and PTHR



A: 5-HT$_7$R nanodisc
B: PTHR nanodisc



Primary antibody: Anti-V5-tag antibody
Secondary antibody: Anti-rabbit IgG-HRP antibody

Figure 3.4 Coomassie blue gel staining and western blot of 5-HT$_7$R nanodiscs and PTHR nanodiscs

3.3 Functional reconstitution of GPCRs into nanodiscs

Nanodisc assembly was carried out with the detergent removal via Bio-Beads. 5-HT_7R and PTHR were successfully embedded into the nanodisc structure. However, successful incorporation of the receptor protein was only possible by finding the ideal molar ratio of receptors to MSP and MSP to lipids (DMPC or POPC). Finding the optimal the molar ratio was a tricky process and different for each membrane protein. The time duration of Bio-Bead application and temperature at which it is applied also seemed to play a role in the success or failure of nanodisc assembly.

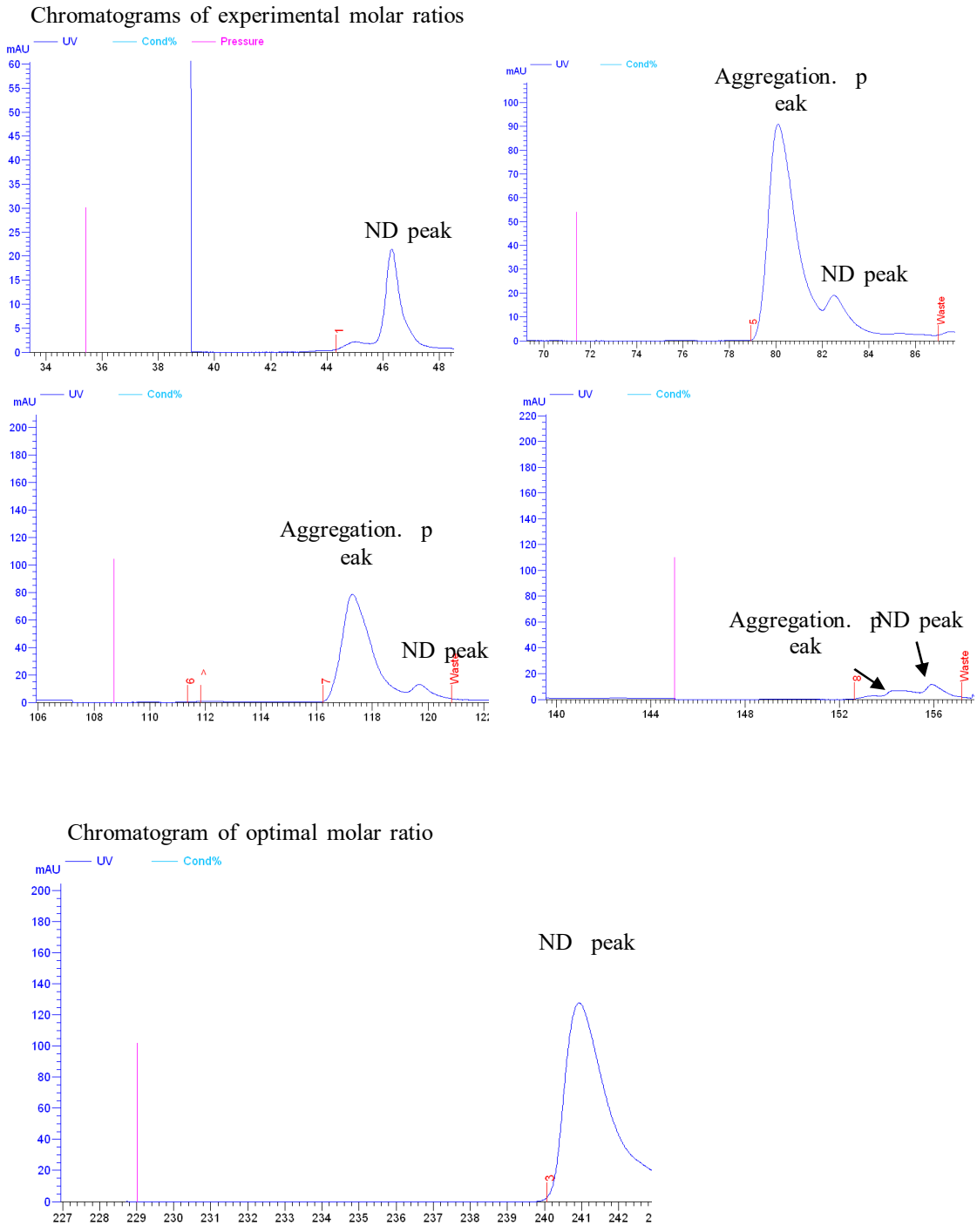
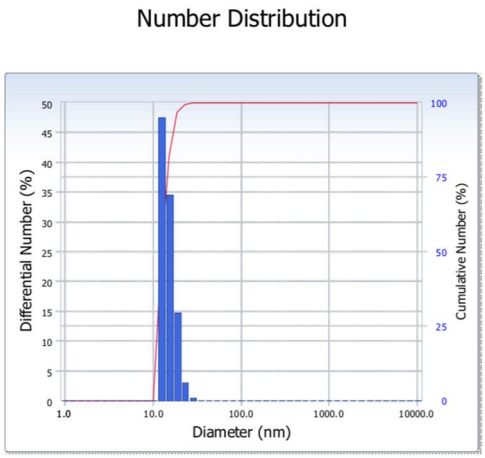
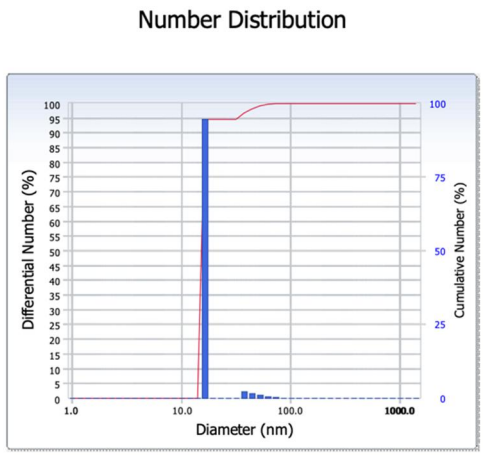


Figure 3.5 Representative chromatograms of SEC results of experimental vs. optimal molar ratio

3.4 Dynamic light scattering

Dynamic light scattering (DLS) was performed on the assembled 5-HT_7 and PTH receptor nanodiscs. The size of the nanodiscs were as reported of other nanodiscs made with MSP1E3D1 (10~20 nm).





Number Distribution

Peak	Diameter (nm)	Std. Dev.
1	16.7	2.5
2	88.9	26.1
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	16.9	4.5

Number Distribution

Peak	Diameter (nm)	Std. Dev.
1	16.4	3.3
2	103.8	26.9
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	16.4	3.5

Figure 3.6 Dynamic light scattering data of 5-HT$_7$R and PTHR nanodiscs

3.5 Tryptophan fluorescence quenching assay

Real-time tryptophan fluorescence of 5-HT_7 and PTH receptor nanodiscs was measured as in **Figure 3.7**. 5-HT_7R responded selectively to one of its ligands, a FDA-approved drug called AS-19. The tryptophan fluorescence was quenched only by a stimulus of AS-19. When stimulated by KDDG-00047, a control substance, beyond a slight change in fluorescence at lower concentrations, the fluorescence did not decrease in a dose-dependent manner. The intrinsic fluorescence of a functional receptor can only be quenched by the selective binding of a ligand to the receptor [15]. The above results demonstrate that functional GPCR nanodiscs were produced.

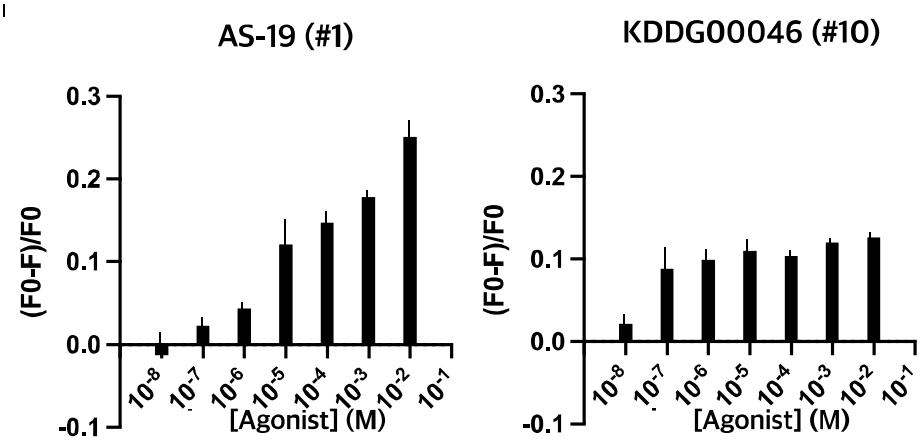


Figure 3.7 Tryptophan fluorescence quenching assay data of

of 5-HT_7 receptor nanodiscs

3.6 Fluorescence polarization assay

Empty (no receptor-embedded) nanodiscs were used as control in comparing the fluorescence polarization to GPCR-embedded nanodiscs. Afterwards, inhibitors, which represent potential drug candidates of the relevant receptors, were added to GPCR/tracer complexes. The fluorescence polarization decreased until saturation, demonstrating that tracers were successfully replaced by inhibitors.

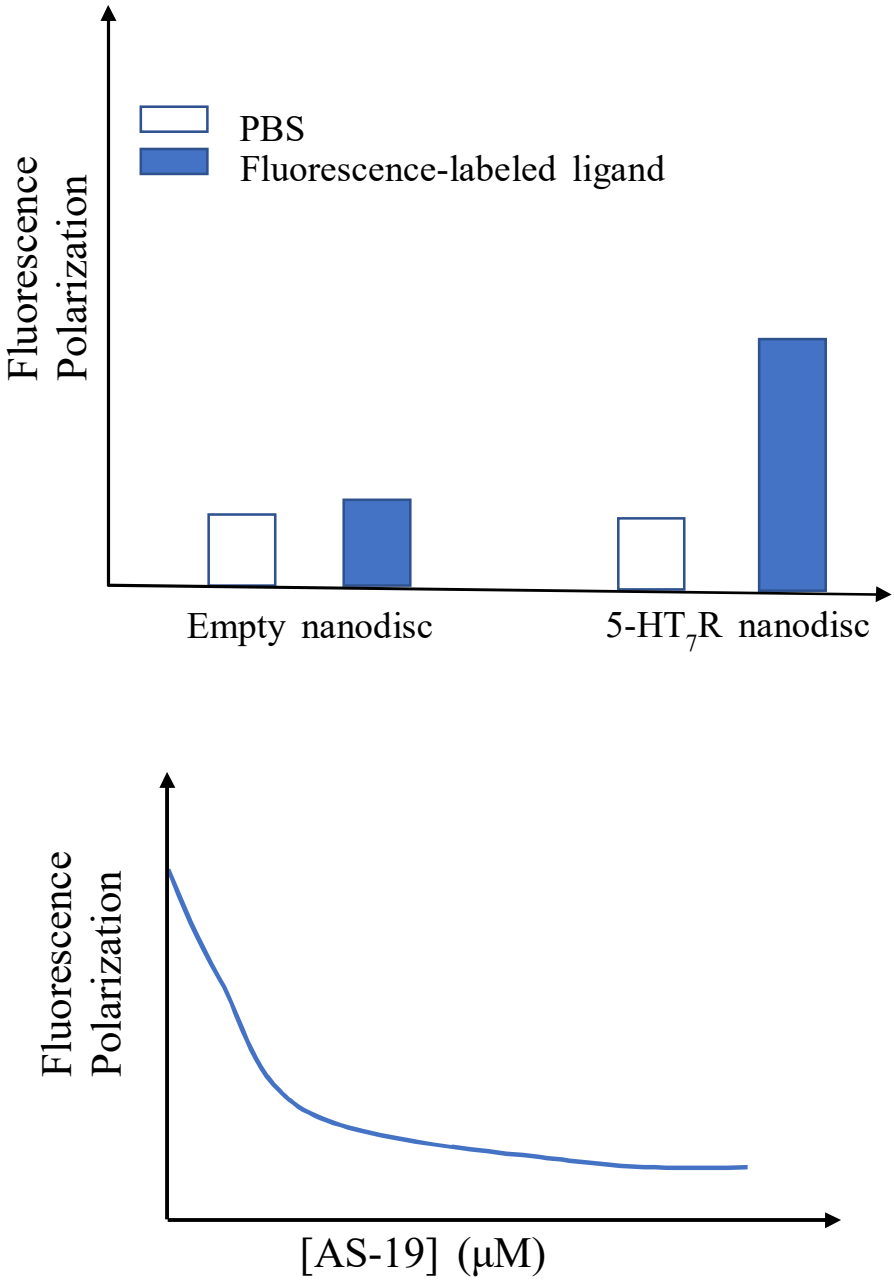


Figure 3.8 Fluorescence polarization assay results with AS-19, a drug target of 5-HT_7R

4. Conclusion

The nanodisc is the optimal method to reconstitute proteins into their native structures. GPCRs, when functionally reconstituted into nanodiscs, can effectively be used for biosensors and drug screening platforms. As GPCRs are fantastic targets for small-molecule drugs, identifying promising receptors that could be targeted for novel drugs for various diseases is important for receptor-based research.

This study presents 5-HT_7 and PTH receptors, which have the potential to be targets for novel small-molecule drugs, and introduces a drug screening platform in the form of the fluorescence polarization assay. To create a drug screening platform, the receptors' plasmids were first cloned. Then, the receptors were expressed in *E. coli* and purified. With the optimal molar ratio of receptors: MSP: lipid, GPCR nanodiscs were assembled and further purified through Nickel-column chromatography and SEC. Examining the chromatograms from SEC allowed the optimization process for increasing the yield of nanodiscs and reducing the amount of protein aggregates.

The staining and blotting data demonstrated that GPCR nanodiscs were purified properly, as the receptors and MSP were found present. The DLS data confirmed that nanodiscs were produced in the expected sizes of MSP1E3D1 nanodiscs which are 10~20 nm. Tryptophan fluorescence quenching affirmed that the produced nanodiscs of correct sizes were functional. These functional and uniform-sized

nanodiscs were then incorporated into the fluorescence polarization assay. The data showed that the nanodisc/labeled-tracer complex is a promising model for discovery of receptor-specific drugs, demonstrating its high selectivity as the potential drug candidates must have a higher affinity than the tracer. Overall, the nanodisc fluorescence polarization assay platform holds great promise for use in discovering novel pharmaceuticals.

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요약 (국문 초록)

G 단백질 연결 수용체 (GPCR)는 인간 유전체 중에 가장 높은 비율을 차지하는 수용체 단백질로, 여러 질병 및 상용화된 약물의 34% 이상이 GPCR 을 타겟할 정도로 중요한 단백질이다. 5-HT_7 수용체 (5-HT_7R)는 세로토닌 계열 수용체 중에 제일 최근 발견됐으며 20 개 이상의 상용화된 약품들이 이 수용체와 관련돼있다. 기능적으로는 수면, 우울장애, 학습, 기억력, 통각 등과 밀접한 관련이 있다. 부갑상선 호르몬 수용체 (PTHR)는 뼈, 신장, 장 등의 기능을 관리하고 골다공증 등의 질병을 완화하는 물질의 타겟으로 사용된다.

하지만 약물 상호 작용 연구등의 목적을 위한 대장균에서의 GPCR 생산은 여전히 한계가 있다. 수용체가 내장된 나노디스크는 수용체/리간드 생물 물리학적 분석을 위한 훌륭한 기법이다. 5-HT_7 과 PTH 수용체를 나노디스크 플랫폼으로 재구축하여 5-HT_7 과 PTH 수용체 리간드에 대해 높은 민감도와 특이도를 자랑하는 스크리닝 플랫폼을 구축하였다. 본 논문에서 두 종류의 GPCR 은 대장균에서 과량생산 되었으며, 수용체, membrane scaffold protein, 지질로 구성된 나노디스크로 재구축하였다. 컬럼 크로마토그래피를 통해 고순도로 정제한 나노디스크를 형광편광 assay 에 적용 시켰다. 수용체를 포함한 나노디스크 기반의 플랫폼은 저분자 약물 스크리닝 기법의 실용적인 방법으로 사용될 수 있을 것으로 기대된다.

주요어: GPCR, 5-HT$_7$R, PTHR, 나노디스크

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