In Situ Reconstitution of the Adenosine A$_{2A}$ Receptor in Spontaneously Formed Synthetic Liposomes

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S *Supporting Information*

ABSTRACT: Cell transmembrane receptors play a key role in the detection of environmental stimuli and control of intracellular communication. G protein-coupled receptors constitute the largest transmembrane protein family involved in cell signaling. However, current methods for their functional reconstitution in biomimetic membranes remain both challenging and limited in scope. Herein, we describe the spontaneous reconstitution of adenosine A$_{2A}$ receptor (A$_{2A}$R) during the de novo formation of synthetic liposomes via native chemical ligation. The approach takes advantage of a nonenzymatic and chemoselective method to rapidly generate A$_{2A}$R embedded phospholiposomes from receptor solubilized in *n*-dodecyl-β-D-maltoside analogs. In situ lipid synthesis for protein reconstitution technology proceeds in the absence of dialysis and/or detergent absorbents, and A$_{2A}$R assimilation into synthetic liposomes can be visualized by microscopy and probed by radio-ligand binding.

The G protein-coupled receptors (GPCRs) represent the largest class of transmembrane receptors found in eukaryotes.[1,2] This superfamily functions in signal transduction involved in numerous physiological processes including sensory phenomena and metabolism.[3] GPCRs recognize a wide variety of structurally diverse ligands (agonists and antagonists) such as hormones, peptides, lipids, nucleotides and neurotransmitters.[4] Besides G proteins, GPCRs may couple with multiple intracellular partners (e.g., arrestins) and undergo endocytosis.[5] Thus, the precise conditions under which GPCR-mediated signaling is processed can be difficult to characterize, particularly with respect to signaling bias or allostery. Reductionist strategies are commonly employed to decouple state or structural determinants that elicit a specific ligand induced signal-response. One such strategy is to study the receptor dynamics in monodisperse detergent micelles. Unfortunately, most membrane proteins, including GPCRs, are unstable in detergent bilayers and display altered (compromised) pharmacological and functional properties.[6] To circumvent these detergent effects, membrane proteins may be reconstituted in stable-biomimetic membranes such as vesicles, reconstituted high-density lipoprotein (HDL) [nanodiscs], bicelles or with amphipoles.[7,8] Although these approaches are powerful and have uncovered fundamental properties of GPCR function, they are quite methodologically cumbersome, requiring chromatography steps to remove detergents. Moreover, structural features normally found in cell membranes such as curvature and polarity are mostly absent. Interpretations based on these methods may overlook the degree to which GPCR signal scaffolding depends on membrane curvature and composition. In this regard, a rapid and robust reconstitution methodology that better mimics the native chemical environment of a whole-cell embedded GPCR would be highly useful.

Recently, we demonstrated the feasibility of using non-membrane forming surfactants, such as lysophospholipid analogs and fatty acyl thioesters, as reactive precursors to generate liposomes and subsequently reconstitute membrane proteins during de novo phospholipid synthesis.[9] However, the lysophospholipids used for protein solubilization are modest detergents for the purification and isolation of membrane proteins.[10] Lysophospholipid critical micelle concentrations (cmc's) range from 4 to 8 μM, which are relatively low, resulting in a greater propensity to form micelles before the surfactant can fully solubilize a membrane protein.[11] Additional challenges exist with the lysophospholipid headgroup. When using ionic or zwitterionic detergents, there is an increased possibility of denaturing a protein of interest, and less success of renaturing and restoring the protein's native function.[12] These drawbacks limit the applicability of our previous methodology in reconstituting more challenging transmembrane proteins like GPCRs. With the goal of developing an efficient in situ reconstitution compatible with GPCRs, we describe here the native chemical ligation (NCL)-promoted incorporation of the adenosine A$_{2A}$ receptor (A$_{2A}$R), a subclass of GPCRs, in synthetic liposomes using novel *n*-dodecyl-β-D-maltoside (DDM) thioester analogs (Figure 1, Figure S1). In situ lipid synthesis for protein reconstitution technology (INSYRT) provides a rapid and selective method for creating GPCR-containing proteoliposomes.

The standard extraction and solubilization of GPCRs involves the use of nonionic alkyl glucoside detergents, specifically DDM, followed by subsequent detergent depletion in the presence of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-
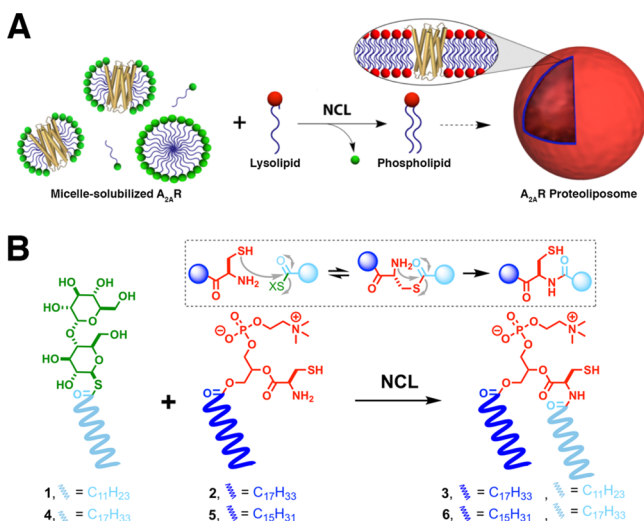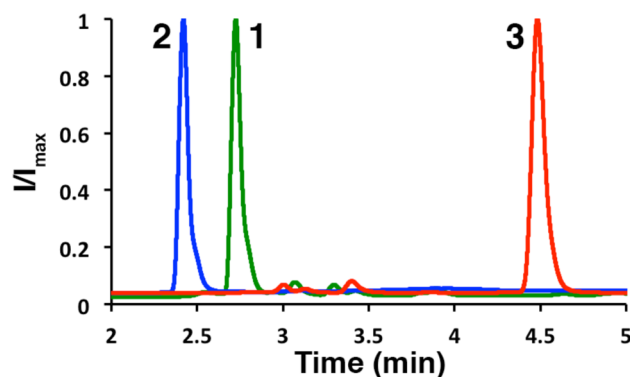
Figure 1. De novo synthesis of phospholipid membranes and concurrent in situ reconstitution of GPCRs. (A) Model for NCL-based phospholipid membrane formation with embedded $A_{2A}R$. (B) Synthesis of phospholipids by NCL reaction of acyl maltose thioesters and cysteine-functionalized choline-based lysophospholipids.

Figure 2. Monitoring phospholipid formation by HPLC/ELSD/MS. ELSD traces corresponding to the purified dodecanoyl maltose thioester 1, lysolipid 2 and phospholipid 3. The retention times were verified by mass spectrometry and the use of known standards.

choline (POPC) (Figure S1).[12,13] The maltoside surfactant coaxes the micellar-solubilized receptors into fusing with a stable lipid-system (e.g., liposomal membrane, HDL nanodisc, planar membrane). DDM forms oblate ellipsoid micelles,[14] which stabilize GPCRs by better encapsulating the bilayer environment, whereas their large micellar size helps in preventing protein−protein aggregation.[15,16] In addition, DDM has a moderate cmc of 170 μM,[17] permitting higher working concentrations than other conventional detergents [e.g., octyl-β-glucopyranoside (OGP) and N,N-dimethyldodecylamine-N-oxide (DDAO)].[18,19] Recognizing the optimal characteristics of DDM, we designed an analog of DDM, the dodecanoyl maltose thioester 1 (Figure 1B, Figures S2 and S3), which can be utilized as both a protein-solubilizing surfactant and a reactive precursor in our NCL reconstitution method (Figure S1).

INSYRT was initially carried out by exchange of DDM for the dodecyl maltose thioester 1 to form micellar-solubilized $A_{2A}R$ (Figure 1A). The protein-solubilized micelles were then reacted with an equal molar ratio of the cysteine-functionalized oleoyl lysophosphatidylcholine 2 (Figure 1B, Figures S2 and S4) through native chemical ligation (NCL) (Figures S5 and S6).[9,20] The reaction afforded phospholipid 3 (Figure 1B, Figures S2 and S5), a synthetic analog of native 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (OPPC). Formation of phospholipid 3 subsequently leads to stable liposome generation and concurrent embedding of $A_{2A}R$ in the membrane. The approach benefits from employing non-enzymatic and chemoselective coupling partners (maltose thioester 1 and lysophospholipid 2) that rapidly react while retaining specificity in buffers, with the only byproduct being the eliminated thiomaltose. The de novo formation of phospholipid also opens up the prospect of rapidly reconstituting the $A_{2A}R$ receptor in liposomes with minimum workflow. The in situ NCL reaction is responsible for the concerted depletion of lysophospholipid and the accumulation of phospholipid in approximately 20 min without the need for additional postworkups or purifications. The reaction was completed as discerned by liquid chromatography (LC), mass

spectrometry (MS), and evaporative light scattering detection (ELSD) (Figure 2, Figure S7). We also observed that the final molar ratio comprising the resulting $A_{2A}R/3$ proteoliposomes is approximately 1:530 ($A_{2A}R$: phospholipid 3). Alternatively, analogous INSYRT experiments using the water-soluble precursors oleoyl maltose thioester 4 and cysteine-functionalized palmitoyl lysophosphatidylcholine 5 also allow efficient formation of phospholipid 6 and subsequent incorporation of $A_{2A}R$ (Figure 1B, Figures S1 to S7).

We next turned to spinning-disk confocal microscopy to visualize liposome morphology and receptor staining using a combination of phase-contrast (Figure 3A) and fluorescence imaging (Figure 3B−D, Figure S8). Fluorescence microscopy was initially carried out with the inclusion of the lipid-staining dye Texas Red DHPE, at a final concentration of 0.5 mol % (Figure 3B). To confirm $A_{2A}R$ was successfully reconstituted in liposomes we labeled purified $A_{2A}R$ with Alexa Fluor 488 N-hydroxysuccinimidyl ester (AF-488 NHS) followed by overnight dialysis and three buffer exchanges to stringently remove any unreacted NHS dye. Immediately prior to the reaction, 2.8 μM fluorophore-modified $A_{2A}R$ was exchanged into 20 mM HEPES buffer, pH 7.4 containing 100 mM NaCl, 20 mM DTT and 1.5 mM dodecanoyl maltose thioester 1. With the addition of lysophospholipid 2, at a final concentration of 1.5 mM, $A_{2A}R/3$ proteoliposome formation was initiated as indicated by the disappearance of both maltose thioester 1 and lysolipid 2, and the formation of phospholipid 3. We observed the colocalization of the fluorescently (AF-488) labeled $A_{2A}R$ with Texas Red DHPE, thus indicating that $A_{2A}R$ is primarily localized to the synthetic phospholipid membranes of the liposomes (Figure 3C).
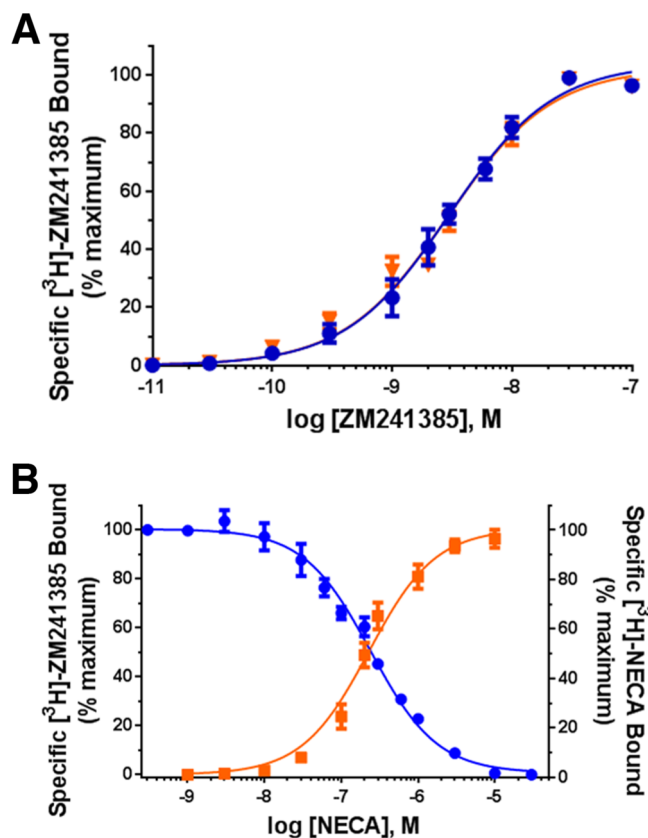
Having shown that $A_{2A}R$ could be spontaneously reconstituted in liposomes, we next sought to determine whether the receptor remains capable of interacting with known orthosteric ligands (Figure 4). This aspect is important when considering that GPCRs in detergent micelles show reduced stability or shifted functional properties and interactions with ligands.[6] Rapid GPCR reconstitution methodologies like the HDL- or nanodics-based approaches allow for increased stability over detergent solubilized receptors and demonstrate equilibrium affinities for ligands comparable to that as observed in membranes.[21] Currently, HDL-GPCR embedded bilayers are the prevailing approach for reconstituting GPCRs.[22] Therefore, we compared the activity of $A_{2A}R$ reconstituted in synthetic membranes using both our in situ NCL-based liposomes and
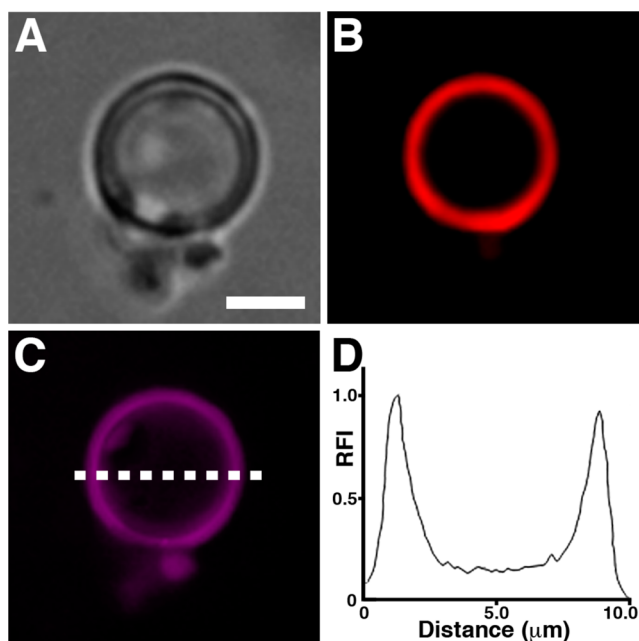
Figure 3. Spinning-disk confocal microscopy of spontaneously reconstituted $A_{2A}R/3$ proteoliposomes. (A) Phase-contrast microscopy of a $A_{2A}R/3$ proteoliposome formed by NCL. (B) Fluorescence microscopy image of an in situ formed $A_{2A}R/3$ proteoliposome, showing the location of the lipid membrane staining dye Texas Red DHPE. (C) Fluorescence image corresponding to an in situ formed $A_{2A}R/3$ proteoliposome, showing membrane staining of $A_{2A}R$, which has been previously labeled with Alexa Fluor 488 dye. The white dashed line corresponds to the intensity profile showed in panel D. (D) Plot profile showing the fluorescent intensity of a typical membrane stained $A_{2A}R/3$ proteoliposome. The diagonally dashed-line in panel C represents the section used to make the histogram. Scale bar denotes 5 μm [RFI: relative fluorescence intensity].

Figure 4. Radiolabeled orthosteric ligand equilibrium experiments with reconstituted $A_{2A}R$. (A) [³H]-ZM241385 saturation curves with in situ formed $A_{2A}R/3$ proteoliposomes (blue line) and $A_{2A}R$-HDL reconstituted nanodiscs (orange line). (B) [³H]-ZM241385 (antagonist)/NECA (full agonist) competition with in situ formed $A_{2A}R/3$ proteoliposomes (blue line) and [³H]-NECA saturation curve with $A_{2A}R$-HDL reconstituted nanodiscs (orange line).

the HDL methodology. Purified $A_{2A}R$ was efficiently reconstituted during the NCL reaction as described earlier, while the HDL reconstitution method was adapted from a previous β_2 adrenergic receptor (β_2AR) HDL incorporation protocol.[8] We initially performed radio-ligand binding assays with [³H]-ZM241385, an antagonist of $A_{2A}R$. Saturation experiments carried out with INSYRT reconstituted $A_{2A}R$ demonstrated a dissociation constant (K_d) of 3.0 ± 0.3 nM ($n = 3$; ±SEM of multiple independent experimental preparations; 2 h incubations at 25 °C) (Figure 4A), which is in close agreement with dissociation constants observed with $A_{2A}R$ reconstituted in HDL nanodiscs [2.9 ± 0.3 nM ($n = 4$; ± SEM of independent experiments)] (Figure 4A) and in isolated cellular membranes.[23] Furthermore, we found that the INSYRT approach yielded a reconstitution efficiency of approximately 30%. We next performed [³H]-ZM241385/5′-(N-ethylcarboxamido)adenosine (NECA) competition and found the inhibitory constant (K_i) to be 150.0 ± 8.7 nM ($n = 3$; ±SEM of multiple independent experimental preparations; 2 h incubations at 25 °C) for the full agonist NECA against in situ reconstituted $A_{2A}R$ (Figure 4B). We also found a K_d of 227.0 ± 25.0 nM ($n = 4$; ±SEM of independent experiments) for specific [³H]-NECA binding in HDLs (Figure 4B), which is in close approximation with literature values.[24]

Radiolabeling experiments showed that the reconstitution of $A_{2A}R$ during INSYRT is similar relative to the HDL reconstitution method based on their respective observed K_d and K_i. Therefore, the advantages of these systems would be application-specific, as the in situ NCL reaction provides compartmentalization compared to the accessibility of the HDL reconstitution (i.e., both sides of the receptor are available for binding). However, for studies of protein−protein or protein−lipid interactions, INSYRT would be desirable because it provides a better mimic to study lateral diffusion and kinetics. For instance, Schuler et al. have recently found that liposomes display lateral thermal expansion coefficients 2-fold higher than in their respective HDL counterparts.[25] These differences are attributed to the HDL boundary lipids being unable to adopt the same phase changes as the lipids in the center of the particle.

In summary, we have shown that $A_{2A}R$, a subclass of GPCRs, can be spontaneously reconstituted in synthetic liposomes resulting from NCL driven membrane formation. Moreover, the key features of INSYRT, orthogonality, rapid reaction rates and biocompatibility, make it a powerful option for reconstituting challenging membrane proteins. The incorporation of $A_{2A}R$ into synthetic lipids demonstrates the utility of this technology to GPCR research. Additionally, the facile one-pot reaction for de novo generation of liposomes is fairly robust and the precursors are straightforward to synthesize. We foresee biotechnological applications that make use of INSYRT in the study of complex membrane proteins or for applications in developing synthetic cells.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b12830.

Detailed procedures, spectral data and Figures S1–S8 (PDF)

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Notes

The authors declare no competing financial interest.

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