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Commentary and Perspective

New lipid membrane technologies for reconstitution, analysis, and utilization of 'living' membrane proteins

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Introduction

Biomembranes play important roles by providing a variety of essential functions in cellular systems such as material transport, signal transduction, and energy production. These functions are achieved by the complex interplay of membrane proteins and lipid bilayers. The human genome sequence project estimated that 20% of the total genome encodes membrane proteins [1]. Due to their important biological functions, over 50% of modern medicinal drugs are designed to target membrane proteins. However, structural and functional analyses of membrane proteins are technically more difficult compared to water-soluble proteins, and a much smaller number of membrane proteins have been characterized. Although most of the biochemical analyses are performed in the aqueous solution, membrane proteins are generally unstable in water and denatured because hydrophobic amino acid residues are exposed to their surface. Therefore, there is a great demand for the development of novel membrane-mimetic systems that can handle the membrane proteins in a native state.

Many attempts have been previously made to provide platforms for the analysis of membrane proteins in a near-native environment by employing artificial membrane systems (Fig. 1). Conventionally, detergent micelles are widely used for the extraction and isolation of membrane proteins, however, micelles often caused destabilization of folded membrane proteins [2]. In addition, residual detergents in the reconstituted membrane is another concern for the detergent-sensitive membrane proteins. To overcome the technical issues of the conventional detergents, various synthetic lipids and amphiphiles have been designed and synthesized for the reconstitution of membrane proteins. Various methods have been developed for constructing artificial membranes having different formats, including vesicles, planar lipid bilayers, nanodiscs, lipidic cubic phases. Cell-sized giant vesicles enable direct

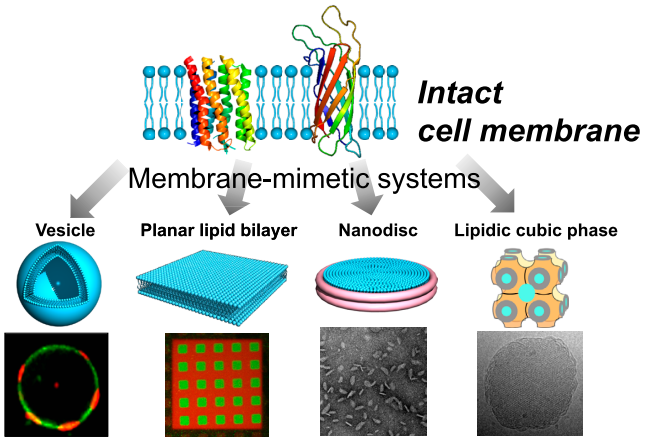

Figure 1 Schematic illustration of the membrane-mimetic systems for the analysis of membrane proteins

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observation of the membrane dynamics by microscopy. The planar lipid bilayer can be applied to the fabrication of lipid membrane array in combination with a micro processing technique that enables the temporal-spatial analysis of multiple membrane proteins. Integration of these new technologies is expected to contribute to understanding the nature of membrane proteins from the biophysics viewpoint. In this article, we briefly introduce the recent progress in the new lipid technologies that enable the reconstitution and analysis of membrane proteins in a 'living' state. These topics are featured in the corresponding symposium at the 58th Annual Meeting of the Biophysical Society of Japan held in September 2020.

Artificial membrane systems that simulate the environment of native membrane

Synthetic lipids or amphiphiles have a potential to provide promising molecular platforms for constructing artificial membranes with native membrane-like properties (Fig. 2). Sonoyama *et al.* employed partially fluorinated phospholipids for the reconstitution of membrane proteins. Bacteriorhodopsin (bR) reconstituted in the membrane formed by di(nonafluorotetradecanoil)-phosphatidylcholine (F4-DMPC) maintained the 2D hexagonal lattice structure that was similar to the form found in the native purple membrane in a wide range of temperature [3]. In contrast, the trimer-to-monomer transformation of bR was induced by the chain melting gel-to-liquid crystalline phase transition when bR was reconstituted in a conventional non-fluorinated phospholipid (dimyristoyl-phosphatidylcholine) membrane. The structural analysis of the F4-DMPC membrane based on the electron density profile of the X-ray diffraction clarified that the partial fluorination maintained the hydrophobic chains of the lipid in an ordered structure even above the chain melting temperature. These results suggested that the rigid molecular packing of hydrophobic core in the lipid membrane is needed to stabilize the bR trimer.

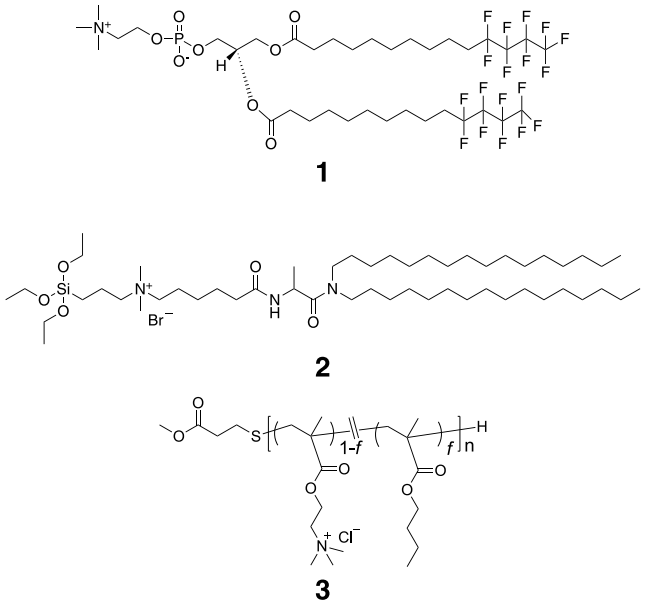

Figure 2 Chemical structures of synthetic lipids and amphiphiles for the artificial membranes. Partially fluorinated phospholipid (F4-DMPC, 1), cerasome-forming lipid (2), nanodisc-forming poly-methacrylate (3)

Conjugation of a cross-linkable moiety to the lipid molecule contributes to enhancing the structural stability of the lipid membrane. Kikuchi *et al.* have developed organic-inorganic hybrid vesicles called 'cerasomes' that display significantly enhanced morphological stability compared to conventional phospholipid vesicles [4]. The cross-linked ceramic-like poly(siloxane) layer was formed on the surface of the membrane by the spontaneous hydrolysis of the alkoxysilane group introduced to the hydrophilic head group of the cerasome-forming lipid in water and following polycondensation between lipid molecules. The higher structural stability of the cerasome enables surface modifications, hierarchical assembly of vesicles, integration on a molecular template, and long-term encapsulation of molecules. The formation of a siloxane layer is a versatile approach to stabilize various lipid assemblies that is applicable not only to the vesicles but also nanodiscs [5] as well as a lipidic cubic phase.

Recently, there has been an increasing interest in lipid bilayer nanodiscs that provides a minimal model membrane in an aqueous solution for the analysis of membrane proteins [6]. The nanodiscs formed by membrane scaffold proteins (MSPs) have been successfully applied to the analysis of a variety of membrane proteins, however, their preparation requires detergents, which are sometimes harmful to membrane proteins. Yasuhara *et al.* designed a nanodisc-forming polymer by employing a poly-methacrylate molecular framework that mimics the amphiphilic nature of membrane scaffold proteins [7]. The addition of the nanodisc-forming polymethacrylate derivative to phospholipid vesicles spontaneously form homogeneous lipid nanodiscs through the fragmentation of the membranes without the aid of any detergents. As another benefit, the polymer-based nanodiscs lack spectroscopic signals that overlap with those of the protein of interest. This nanodisc technology was previously applied to the analysis of the oligomer formation by amyloidogenic peptides such as human islet amyloid polypeptide [7] and amyloid-β peptide [8] in the membrane.

Cell-sized giant unilamellar vesicles (GUVs) enable *in situ* observation of dynamic morphological changes of lipid membranes by light microscopy. Yamazaki *et al.* extensively studied the interaction of antimicrobial

peptides, cell-penetrating peptides, and pore-forming toxins with lipid membranes by the microscopic observation of single GUVs. Magainin-2 is a class of antimicrobial peptide, which is known to form a pore structure in a bacterial membrane to induce the membrane permeation. The fluorescence microscopy of GUVs can simultaneously monitor the binding of the dye-labeled Magainin-2 to the membrane and the leakage of the entrapped fluorescent marker induced by the peptide in a time-dependent manner. They have observed that the Magainin-2 forms pores in the GUV membrane and induced leakage of the entrapped marker stochastically. The kinetic analysis of the leakage clarified that the pore formation by Magainin-2 was influenced by various membrane properties such as lipid composition [9], membrane stretching [10], as well as membrane asymmetry [11].

The heterogeneity of the lipid composition in the membrane plays an important role in the function of native biomembranes as proposed in the lipid raft hypothesis. Reconstitution of the domain-forming membrane is expected to clarify the mechanism of the membrane heterogeneity in the biophysical viewpoint. Takiue *et al.* have studied the physicochemical mechanism of the domain formation by various amphiphiles at the interface by focusing on the line tension along the domain boundary [12]. The ternary mixture of a saturated phospholipid, unsaturated phospholipid, and cholesterol has been known to reconstitute the phase separating membrane with lipid raft-like liquid-ordered and liquid-disordered domains [13]. The microscopic observation of the raft-mimicking ternary lipid GUVs is expected to clarify the role of line tension along the boundary of the lipid raft.

Functional Reconstitution of membrane proteins

Reconstituting membrane proteins into a model membrane in a functional state has a long history of development dating back to proteoliposomes and black lipid membranes, which played critical roles in elucidating the functions of membrane proteins [14]. Recent studies have extended these technological bases to reconstitute membrane proteins into a more complex and controlled model systems (Fig. 3). There are two conceptually different approaches, reconstitution of detergent-solubilized proteins, and reconstitution of cell-derived membrane vesicles. The latter approach is exemplified by the incorporation of cell membrane blebs, vesicles induced by the chemical treatment of cell membranes, into a substrate-supported model membrane [15]. This approach can reconstitute native membrane proteins without extensive purification steps that is generally advantageous for reconstituting diverse proteins and their complexes, which are often difficult to purify and reassemble. Yoneda *et al.* recently reconstituted the whole thylakoid membrane from spinach chloroplast into a patterned model membrane on a glass substrate to reproduce the photosynthetic activities [16]. Thylakoid membrane reconstituted into a planar model membrane provides a versatile platform for studying the two-dimensional membrane organization in photosynthesis.

Reconstituting detergent-solubilized proteins has the advantage that one can study purified membrane proteins in a membrane having well-defined lipid compositions. In this approach, however, solubilizing membrane proteins with detergents while keeping their native structures and activities has been a major technical hurdle. Recent advances in the development of novel detergents and protocols to solubilize membrane proteins, in conjunction

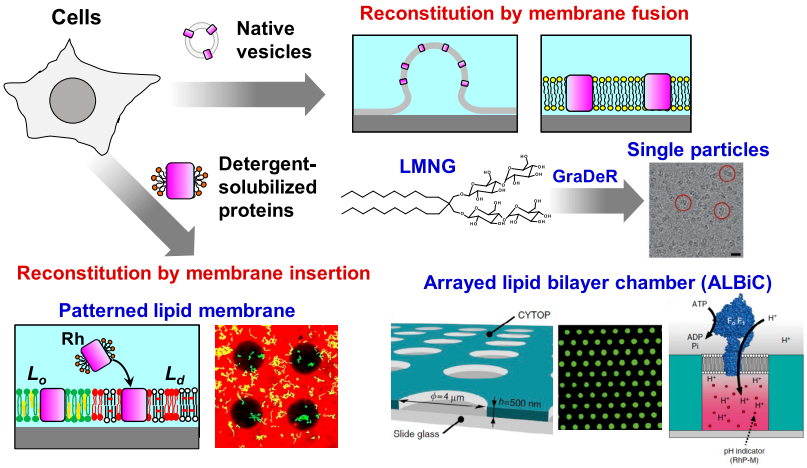

Figure 3 Schematic of the approaches applied to incorporate membrane proteins into model membranes. The images were adapted with permission from Hauer *et al.*, 2015, Tanimoto *et al.*, 2015, and Watanabe *et al.*, 2014.

with the rapid advances in the structural determination by the cryo-electron microscopy (cryo-EM), have significantly pushed the boundary. For example, laurylmaltose-neopentyl glycol (LMNG) can stabilize membrane proteins even below the critical micelle concentration (cmc), owing to its extremely slow off-rate [17]. The group of Gerle has developed a novel method called gradient-based detergent removal (GraDeR), in which free LMNG micelles and monomers could be effectively removed, resulting in monodisperse and stable membrane protein particles. This method was applied to obtain highly pure and stable F-ATP synthase from bovine hearts to elucidate the molecular mechanism of the mitochondrial megachannel (MMC)/permeability transition pore (PTP), which dissipates the proton gradient in a Ca^{2+} dependent manner and is a key effector of cell death [18].

LMNG has been used also for reconstituting membrane proteins into planar membranes. Watanabe *et al.* developed a planar membranes array formed on a micro-fabricated substrate having numerous chambers (arrayed lipid bilayer chamber system: ALBiC) [19]. Owing to the small sizes of the chambers, the width of several micrometer and the volume of femtoliter, ALBiC can detect movement of molecules and ions in the vertical direction with a heightened sensitivity. Membrane proteins such as F_0F_1-ATP synthase and phospholipid scramblase (Transmembrane protein 16F: TMEM16F) have been reconstituted and their activities were measured with the single molecules sensitivity [20]. Reconstituting detergent-solubilized proteins is applicable in a wide variety of membrane formats. Tanimoto *et al.* reconstituted a G protein-coupled receptor (GPCR) in the vertebrate retina, rhodopsin (Rh) photoreceptor, and its cognate G protein transducin (Gt) into a patterned membrane having liquid-ordered and liquid-disordered regions to evaluate their affinities in different lipid phases [21]. The obtained distributions have implications to the functional importance of protein localization in the biological membrane through interactions with lipids.

Conclusion and perspective

Model membranes have contributed to our understanding of the biological membrane since the development of Langmuir monolayer. In recent years, new types of model membranes have been developed in combination with micro-fabrication techniques and sensitive analytical/imaging methods. One important objective of the model membrane is to study the functions of membrane proteins in a controlled, native-like lipid environment. To this end, unique lipid matrices, detergents, and techniques to reconstitute membrane proteins have emerged. These developments are currently underway and we can hope to witness rapid progress in constructing model membranes with functional membrane protein to simulate diverse membrane functions and obtain quantitative information of the molecular mechanisms.

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