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Current Opinion in Biotechnology

Synthetic (polymer) biology (membrane): functionalization of polymer scaffolds for membrane proteins

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A plethora of polymer-based scaffolds have been designed to facilitate biochemical and biophysical investigation of membrane proteins, with a common goal to stabilize and present them in a functional format. In this review, an up-to-date account of such polymer-based supports and incorporation methodologies are presented. Furthermore, conceptual and imminent technological advances, with associated technical challenges are proposed.

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Current Opinion in Biotechnology 2017, **46**:51–56

This review comes from a themed issue on **Nanobiotechnology**

Edited by **Benjamin G Davis** and **Christopher Serpell**

For a complete overview see the Issue and the Editorial

Available online 7th February 2017

http://dx.doi.org/10.1016/j.copbio.2016.10.012

Introduction

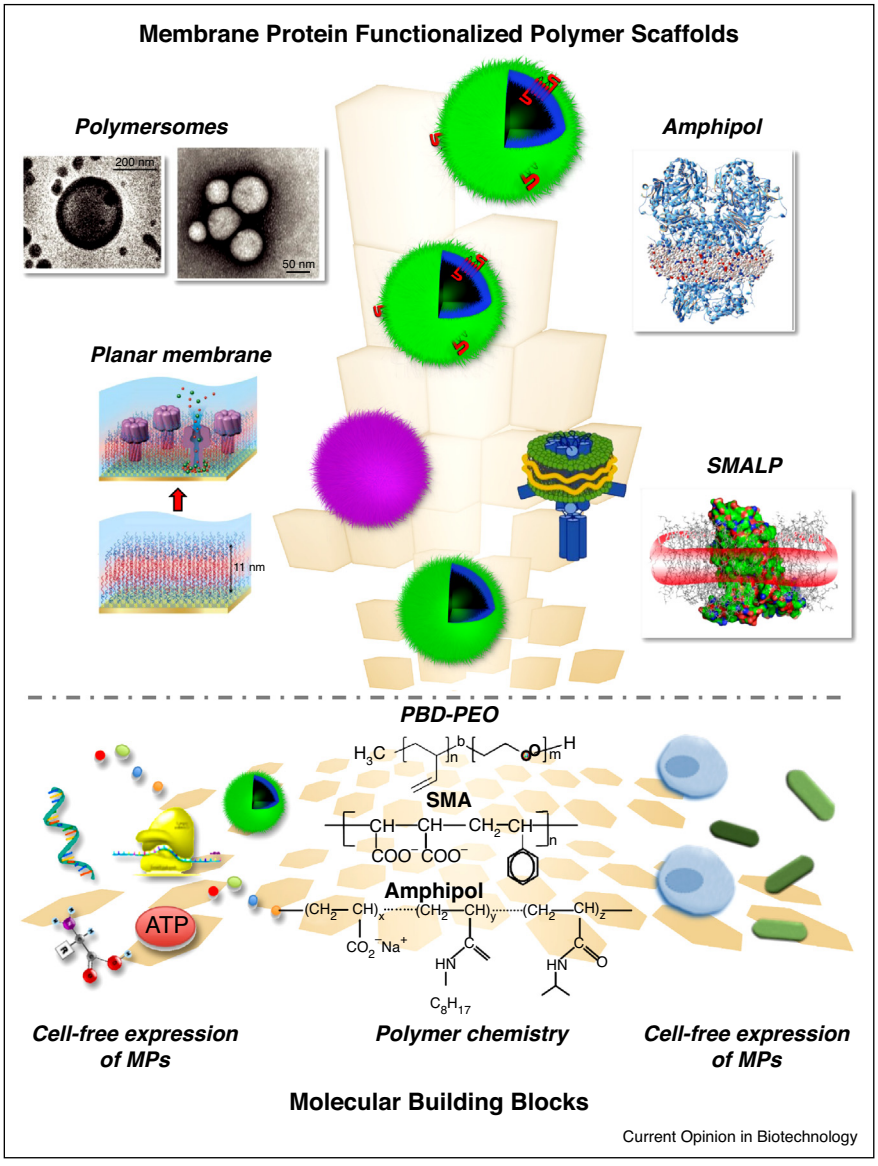
Membrane proteins (MPs) are key functional units of cell membranes, constituting about 20% of the total human genome coding proteins [1]. MPs modulate intracellular signaling networks in response to extracellular cues, thus, playing an indispensable role in cell communication. Hence, it is not surprising that the majority of approved drugs (60%) target different classes of MPs [1]. Successful development of such drug molecules relies heavily on detailed and precise structural information of MPs that correlates with its function [2]. However, obtaining cognate structure-function information is a daunting task, which is well reflected in the literature, with only a few MP structures have been reported thus far [3]. Unlike soluble proteins, MPs go through a series of intricately regulated co- and post-translational processes from the endoplasmic reticulum (ER) through the Golgi to the plasma membrane [4] to achieve proper folding. Most often, these folded structures are characterized by (i) alternating hydrophobic transmembrane regions that consist mostly of alanine and glycine residues, (ii) closely packed transmembrane helical regions, and (iii) deeply buried interfacial surface [5]. Once removed from their amphipathic environment, these folded proteins are mostly perturbed, resulting in a loss of native function. However, for biochemical and biophysical characterization, it is necessary for MPs to be isolated from their native amphipathic environments and introduced into an artificial environment that is expected to preserve their structural integrity and function. This is often accomplished by the use of detergents [6] and reconstituted in liposomes [7]. Liposomes are vesicular bilayer structures assembled from natural or synthetic phospholipids and are reminiscent of cell membranes [8]. Despite being the most commonly used system for MP reconstitution, liposomes suffer from several shortcomings including extreme sensitivity to external perturbations, limited shelf-life, and short *in vivo* circulation time [9]. To overcome these drawbacks, self-assembling amphiphilic polymer-based vesicles have been developed [10]. Amphiphilic polymers, with high chemical diversity, exhibit highly tunable membrane properties, (e.g., bilayer thickness, hydrophobicity, rigidity and permeability) [11] stability, and longer circulation time *in vivo* [10]. Furthermore, advances in polymer synthesis have enabled chemists to mimic and fabricate other scaffolds employed in preserving the structural integrity of MPs, which have garnered increased interest over the past few years (Figure 1).

Here, we review advances in polymer-based supports and recent methodologies that are employed to incorporate MPs into an environment conducive for its biophysical and biochemical characterization.

Methodologies of membrane protein incorporation into polymer membranes

To facilitate the investigation of MPs in an artificial environment, they are incorporated into polymer membranes mainly via two methods, that is reconstitution by detergent removal [17] and co-translational insertion [18]. For the reconstitution method, MPs are isolated either from endogenous cells or recombinant protein expression cells (i.e., bacterial, insect and mammalian cells). In both cases, the MPs are isolated via detergent-mediated membrane solubilization, resulting in MP-detergent complexes. Subsequently, the MPs are reconstituted into

Figure 1



An overview of polymeric supports presenting membrane proteins that are assembled from various building blocks (polymer molecules, MPs expressed by different methods). Modified with permission from Ref. [12•] (Copyright 2016 Portland Press Limited), Ref. [13] (Copyright 2003 Birkhäuser-Verlag Basel), Ref. [14] (Copyright 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim), Ref. [15] (Copyright 2012 American Chemical Society), and Ref. [16] (Copyright 2011 John Wiley & Sons, Inc.). SMA: styrene maleic anhydride. PBD-PEO: diblock polymer polybutadiene-polyethylene oxide.

supporting vesicular or planar membranes by detergent removal. The uncertainties and technical difficulties concerning the use of detergents to solubilize MPs have been well documented. These include the inability to reproduce the complex native lipid environment, incompatibility with downstream analyses, and structural alterations [19]. As a result, MPs are selected primarily on the technical feasibility of obtaining a reconstituted MP rather than its functional aspect [20]. Recently, a detergent-free solubilization method using styrene maleic anhydride (SMA) has been explored, in which MPs along with a nanosection of native membrane are extracted and stabilized in a disc-shaped SMA-encircling lipid particle (SMALP) [21].

Co-translational insertion is another method employed in incorporating MPs into membranes. This method relies on the simultaneous mRNA translation and membrane insertion during cell-free protein synthesis (CFPS) [22]. Unlike endogenous and recombinant cell expression, CFPS is independent of live cells. Instead, cell lysates containing translational machinery (ribosomes, translation

factors and metabolic enzymes) is supplied along with essential elements (DNA or RNA template of the protein of interest, and RNA polymerase cofactors), and energy resources for protein synthesis [23•]. Being an open reaction system, CFPS allows extensively modified reactions, and addition of supplementary components such as detergents and lipid- or polymer-based supports facilitate MP folding and stabilization [19,24–26,37]. However, addition of detergents is not always possible as they may inhibit the translational machinery in CFPS. Moreover, cellular folding mechanism cannot be fully replicated with translational machinery provided by CFPS. Despite these limitations, successful cases have been reported with liposomes [27], nanodiscs [28] and polymersomes [14].

Repertoire of polymer supports for membrane proteins

A plethora of polymer-based MP supporting platforms have been developed over the last two decades. These include polymersome (liposome, being the lipid equivalent), polymer-based planar membrane (equivalent to supported lipid bilayer), amphipol (like amphipathic surfactant) and SMA (alternative to apolipoprotein) summarized in Table 1. Meier et al. pioneered the successful incorporation of MPs into polymer membranes, by isolating bacterial porins (OmpF and LamB) with detergents and reconstituting them in black PMOXA-PDMS-PMOXA membrane [29•]. Later studies have largely focused on prokaryotic MPs, including transporters, porins, channels, and enzymes (Table 1).

Polymersomes offer excellent scope for compartmentalization, closely mimic cell membranes, and are compatible for downstream applications [11]. Aquaporin, a MP channel for water and small solutes, has been successfully embedded into polymer membranes to develop aquaporin-based biomimetic polymeric membranes (ABPMs). Because of their superior stability and water channel activity when embedded in the polymer membranes,

Table 1

Overview of polymer supports for membrane protein incorporation and related biophysical and biochemical assays

Membrane supports	Method of incorporation	Protein source	Protein class	Assays	Ref.
Polymersomes	Reconstitution	Cell-based expression	Channel (aquaporin Z)	EM, AFM, SFLS	[38]
			Channel (aquaporin Z)	EM, EA, SFLS	[39]
			Transporter (BR) Enzyme (ATP synthase)	EM, EA	[40]
			Porin (maltoporin, OmpF)	EM, EC	[29•]
	Co-translational incorporation	Cell-free expression	Junction protein (Claudin-2)	EM, SPR, FS	[42]
			Light-harvesting complex II (LHC II)	EM, PDA, SPFS	[43]
			GPCR (dopamine receptor D2)	FC, FM	[14]
			GPCR (chemokine C-X-C receptor 4)	SPR, RL	[44••]
Planar	Reconstitution	Cell-based expression	Channel (MloK1)	AFM, ATR-FTIR, FM, EC	[41]
			Photosynthetic complex (RCs)	EM, SAXS, FS, UV, EA	[45]
			Transporter (proteorhodopsin)	DSC, EM, SAXS, RS, NMR	[46]
SMA	Direct solubilization	Cell-based expression	GPCR (Adenosine A2a)	RL, CD	[47]
			Channel (KcsA)	EM, CD, FS, PC	[21]
			Transporter (AcrB)	FP, EM, AUC	[48]
			Cell division protein (ZipA)	EM, SEC, CD	[49]
			Enzyme (Respiratory Complex IV)	EM, SEC, EA	[50]
	Preformed proteoliposomes/direct solubilization		GPCR (MT1R, GHS-R1a)	FS, SEC, RL, EA	[51]
	Preformed proteoliposomes	Cell-based expression	Transporter (BR), enzyme (PagP)	SEC, EM, CD, EA, UV	[52•]
Amphipol	Reconstitution	Cell-based expression	Transporter (BR), porin (OmpF)	SPR, SEC	[35]

Abbreviations:
Protein targets: GPCR: G-protein coupled receptor; OmpF: outer membrane protein F; LHC II: light-harvesting complex II; MloK1: K+ channel from *Mesorhizobium loti*; RCs: photosynthetic reaction centers; KcsA: K+ channel from *Streptomyces lividans*; ZipA: Z interacting protein A; PagP: PhoP-activated gene; MT1R: melatonin receptor; AcrB: acridine resistance protein B; GHS-R1a: growth hormone secretagogue receptor; BR: bacteriorhodopsin.
Characterization techniques: AFM: atomic force microscopy; ATR-FTIR: attenuated total reflection Fourier transform infrared spectroscopy; AUC: analytical ultracentrifugation; CD: circular dichroism; DSC: differential scanning calorimetry; EA: enzymatic activity; EM: electron microscopy; EC: electrical conductance measurement; FC: flow cytometry; FM: fluorescence microscopy; FP: fluorescence polarization; FS: fluorescence spectroscopy; MS: mass spectroscopy; NMR: nuclear magnetic resonance spectroscopy; PC: patch clamp; RL: radioligand binding assay; PDA: protease digestion assays; RS: Raman spectroscopy; SAXS: small-angle X-ray scattering; SEC: size-exclusion chromatography; SFLS: stopped-flow light-scattering; SPR: surface plasmon resonance; SPFS: surface plasmon enhanced fluorescence spectroscopy; TS: thermal stability assay; UV: UV absorbance.

ABPMs have found applications in water purification and desalination [30•].

A different polymer support system using a polyacrylate-based polymer (amphipols), developed by Popot et al. [31•], has gained traction, showing stable incorporation of various proteins such as cytochrome b6f [15], GPCRs [32], Ca^{2+}-ATPase [33], major outer MP (MOMP) [34], and mitochondrial supercomplex [16], as well as bacteriorhodopsin (BR) and OmpF [35], more recently (Table 1). When it comes to two-dimensional surfaces, supported polymer bilayers (SPBs) and polyhistidine-bearing amphipols provide an alternative for direct MP incorporation. Similar to supported lipid bilayer (SLB), SPB maintains mechanical stability and enables the topology of MP incorporated surfaces to be probed [36]. Despite their versatility, these methods suffer from a major setback stemming from the use of detergents [17].

Knowles et al. reported bacteriorhodopsin (BR) isolation by direct extraction from liposomes or biological membranes using SMA, thus replacing conventional apolipoprotein-based lipid nanodisc [20]. This has been extended for A2AR [47] and K^+ channel [21] without loss of functionality (Table 1). The advantage of being detergent-free throughout the experimental workflow makes SMA a facile one-step technology despite its limitations, such as only one protein is extracted on average per disc and potential precipitation at acidic pH. Furthermore, SMA being a chelator, can interfere with experiments requiring high concentrations of divalent cations.

Alternative systems and future outlook

Polymersomes, with highly tunable properties, can significantly influence its overall membrane architecture, and such structures are yet to be fully explored for MP studies. Furthermore, it has been demonstrated that polymersomes can undergo out-of-equilibrium shape transformations akin to liposomes [53]. All of which suggests that MP incorporation into polymer-based membrane supports, benefit not only from the inherent physicochemical advantages of polymers, but also a native-like environment for the incorporated proteins.

The key to the success of many artificial support systems lies in its ability to mimic cellular membrane structure and composition, which has already been demonstrated by the applications of nanodisc, polymersomes and SMA. Hence, blending polymers and lipids could create mechanically robust systems with long shelf-life and tunability, yet present optimal native-like environments for different MPs.

As highlighted in the previous section, the advantages of CFPS in MP production, especially the ability to supplement components such as lipid-based systems can be further developed. One could potentially marry the versatile polymer chemistry with the benefits that CFPS provides by simply supplementing the translation cocktail with polymer-based systems. In fact, the feasibility of this strategy has been shown for several proteins including dopamine receptor D2 [14], Claudin-2 [54], and C-X-C chemokine receptor type 4 (CXCR4) [44••] in polymersomes composed of di- and triblock copolymers. Thus, we envisage existing polymer-based CFPS to be further developed by supplementing CFPS with the expanding repertoire of polymer-based membrane supports.

To exploit the full potential of polymer-supported MPs, several fundamental questions remain to be addressed, such as the understanding of the CFPS translation process in the presence of polymersomes (or other polymer support systems) and its influence on the co-translation of MPs. Another aspect is to preserve the native folding and functionality of MPs in a cell-free environment during reconstitution. Although detergents have been the choice to mediate reconstitution, it remains a challenge for incorporation of many MPs into polymer (or even lipid) membranes. Thus, alternative strategies such as the addition of chaperones with novel polymer molecules may be adopted. Furthermore, some MPs function as a molecular switch on the cell membrane, and to study downstream cascade effects of such a switch, requires more reliable polymer-based membrane support than what is currently available.

Moving forward, with the emergence of all these different polymer supports, one could envision solving the structure and function of complex mammalian MPs. In this context, GPCRs, being one of the difficult targets, is currently the focus of many studies. However, only a limited number of such targets have been explored to date [55••]. In our opinion, these recent developments are a good starting point towards exploiting novel yet challenging MP targets in a truly interdisciplinary approach.

Funding

This work was supported by Academic Research Fund (AcRF) Tier 1 Grant and the School of Materials Science and Engineering, Nanyang Technological University.

Acknowledgement

We thank Sushanth Gudlur for critical reading of the manuscript.

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