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Engineering supported membranes for cell biology

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Abstract Cell membranes exhibit multiple layers of complexity, ranging from their specific molecular content to their emergent mechanical properties and dynamic spatial organization. Both compositional and geometrical organizations of membrane components are known to play important roles in life processes, including signal transduction. Supported membranes, comprised of a bilayer assembly of phospholipids on the solid substrate, have been productively served as model systems to study wide range problems in cell biology. Because lateral mobility of membrane components is readily preserved, supported lipid membranes with signaling molecules can be utilized to effectively trigger various intercellular reactions. The spatial organization and mechanical deformation of supported membranes can also be manipulated by patterning underlying substrates with modern micro- and nano-fabrication techniques. This article focuses on various applications and methods to spatially patterned biomembranes by means of curvature modulations and spatial reorganizations, and utilizing them to interface with live cells. The integration of biological components into synthetic devices provides a unique approach to investigate molecular mechanisms in cell biology.

Keywords Supported membranes · Lipid bilayer · Immunological synapse · Membrane patterning techniques · Spatial mutation · Membrane curvature modulation

1 Introduction

In biology, the lipid bilayer membrane was first identified as the barrier defining the interior and exterior of cells. As our understanding of cell membranes developed over the years, it has become clear that the complex liquid interface provided by membranes also serves as a primary platform for cellular signal transduction machinery. Indeed, nearly all molecular life processes involve membranes at some point. The basic structural component of cell membranes, the lipid bilayer, has been known to be a two-dimensional fluid since the 1970s [46]. Rather than a homogeneous fluid sheet, lipid membranes in the cell exhibit heterogeneity and flexibility both in compositions and organizations [35]. Now, the spatial organization and morphology of cellular membranes and membrane components are widely known to mediate numerous biological functions [12, 21, 30, 51, 52].

The cellular membrane creates an environment with many unique characteristics for both self-assembled and actively driven molecular sorting processes. Supported lipid membranes, formed by spontaneous fusion of small lipid vesicles onto a hydrophilic surface such as silicon oxide [3], exhibits similar two-dimensional fluidity in vitro as natural cell membranes and has been broadly utilized as a model system to study membrane organizations [4, 13, 21, 29, 31, 39, 42, 49, 50]. Lipids and membrane-linked proteins in the supported membrane can diffuse freely over macroscopic distances with lateral diffusion coefficients generally on the order of $1 \mu\text{m}^2/\text{s}$ [22, 43, 48]. As the

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chemical composition of supported membranes can be easily manipulated, the model membrane system creates many possibilities to represent biocompatible interfaces with controllable physical properties, such as electrostatic charges [17, 28], surface density of molecules [29, 40], and degree of molecule clustering [1, 23].

Another salient characteristic of the support membrane is its compatibility to merge diverse research techniques, such as micro/nano-fabrication and fluorescence microscopy. Solid supported membrane substrates can be spatially patterned via modern techniques [22, 27, 34, 39, 45]. By fabricating barriers on the substrate, typically thin metal lines, supported membranes can be partitioned into different regions while membrane components are locally confined. Direct membrane patterning can also be achieved by UV/ozone photolithography which directly removes lipid membrane [47, 58]. Concurrently, underlying substrates can be fabricated with three-dimensional features and create curvature modulation of supported membranes [42, 56]. Combined with biocompatible surface chemistry techniques, signaling molecules can be represented along with patterned supported membranes, too. Spatial and curvature-patterned supported membranes readily provide distinctive experimental setup to investigate many research aspects in cell biology, such as immunology and cancer biology.

2 Supported membranes and immunological synapses

The immunological synapse between T cell and antigen-presenting cell (APC) involves large scale spatial sorting of membrane proteins in concert with intracellular T cell signaling activity. Previously, functionalized supported membranes have been demonstrated to effectively trigger T cell activations and form hybrid immunological synapses. APC membrane proteins, including antigenic peptide loaded major histocompatibility complex II (pMHC) and intercellular adhesion molecule 1 (ICAM-1) can be chemically attached to lipid membranes and preserve two-dimensional mobility. As the T cell is engaged, T cell receptor (TCR) ligates with mobile pMHC and together form central supramolecular activation complex (c-SMAC) in the immunological synapse. Although biochemical interactions between molecules in immunological synapses have been extensively studied for many years, hybrid immunological synapses provide unique advantages in the study of spatial redistribution of membrane proteins in live T cells.

The lateral mobility of molecules in the supported membrane can be controlled by barriers fabricated onto the underlying substrate. Nanometer scale metal grids, such as thin chromium lines, physically partition supported membranes into separated membrane corrals. Molecules diffuse and assemble normally within each corral but cannot hop

across the barriers. When these patterned supported membranes engage with living cells, cell surface receptors whose cognate ligands are in the supported membrane become subject to the same constraints on molecular movement. In turn, other signaling molecules inside the cell that associate with these receptors also follow the prescribed organization. In this way, it is possible to control the spatial organization of the signal transduction machinery for a specific signaling pathway in living cells. We have referred to such manipulations as spatial mutations; essentially chemically identical cells differing only by their molecular spatial organization are created.

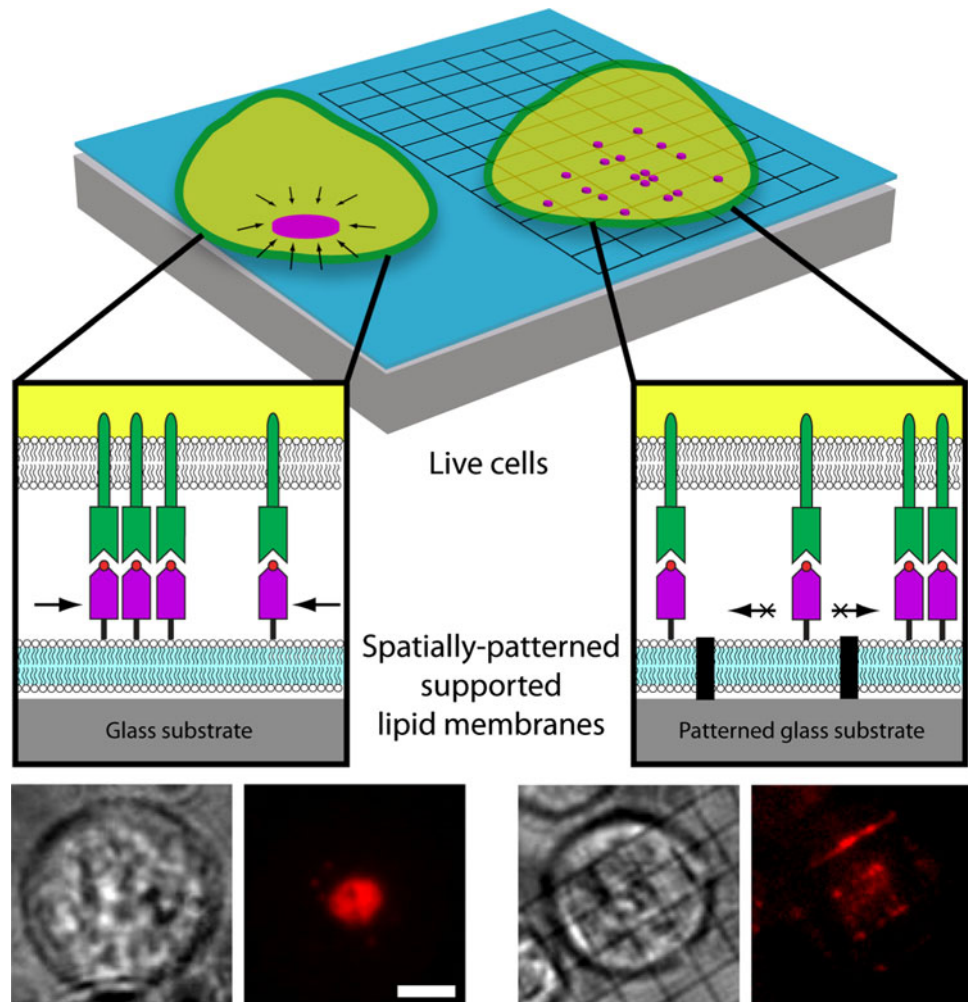
The spatial mutation strategy has proven useful in studies of molecular spatial organization within immunological synapses (Fig. 1). For example, during immunological synapse formation, T cell receptors (TCR) first bind to their cognate ligands, antigenic peptide MHC, and assemble into microclusters. The formation of these microclusters is critical to TCR signaling and, unlike solid substrate display strategies [11], supported membranes allow this type of clustering to occur. At sufficiently high antigen level, TCR microclusters are subsequently transported radially towards the junction center resulting in a bull's-eye pattern with TCR-MHC in the middle and other adhesion molecules forming peripheral rings. It is this pattern that was first named the immunological synapse [18]. Naturally, there was much interest in the role, if any, such a large scale pattern had in signal transduction processes. Using the spatial mutation method in its debut, Mossman et al. [39] demonstrated that TCR clusters were indeed signaling differently if they were physically trapped in the periphery of the cell. Since this first introduction, various forms of the technology have been applied to other aspects of the T cell synapse [9, 57].

Another type of supported membrane patterning has been achieved in the context of the immunological synapse using photo-releasable agonist peptides. In this case, the identity of the agonist peptide, to which the T cell will respond, is masked with a photosensitive chemical moiety 6-nitroveratryloxycarbonyl (NVOC). Irradiations with near-UV light restore agonist activities by photochemically cleaving NVOC protecting group [10, 25]. The ability to photoactivate protein functions on the supported membrane enables both spatial and temporal manipulations to investigate underlying mechanisms of immune synapse assembly and signaling.

3 Other juxtacrine signaling: EphA2–EphrinA1 in cancer biology

Another signaling system that shares the juxtacrine configuration of receptors on one cell membrane with their

Fig. 1 Spatial patterning of fluid supported membranes and applications in live T cell immunological synapses. Various methods have been developed to locally confine two-dimensional mobility in supported membranes. Here, passive grid barriers on the glass support are fabricated by electron-beam lithography and provide lateral confinement of membrane components. By fluorescence microscopy, the bull's-eye protein pattern of the immunological synapse in live T cell is altered by the presence of grid barriers on supported membrane (*right panel*). Scale bar 5 μm



membrane-bound ligands on an apposed cell membrane is EphA2–EphrinA1. EphA2 is a receptor tyrosine kinase and is a member of the large family of Eph receptors, which collectively govern many aspects of cellular assembly into tissues [33, 38]. Upon ligation with endogenous EphrinA1 on the plasma membrane of apposed cells, activated EphA2 can initiate various signal pathways and misregulation of these pathways contributes to cancer metastasis. It has been reported that naturally monomeric EphrinA1 in solution can bind to EphA2 receptors, but fails to trigger EphA2 signal cascades unless it is artificially linked into dimers [8]. In spite of this shortfall, artificial EphrinA1 dimers have been widely used to study the EphA2 signaling system.

Display of EphrinA1 on supported membranes provides a great experimental platform to study the Eph signaling with live cells. Notably, mobile EphrinA1 on supported membranes can effectively trigger EphA2 activation in human breast cancer cells. The lateral mobility of EphrinA1 on supported membranes also reveals dynamic reorganizations of EphA2/EphrinA1 clusters during signaling.

While the underlying mechanism of EphA2/EphrinA2 macroscopic protein pattern formation is still unclear, application of the spatial mutation technology to this system has enabled investigation the role of spatial redistribution in signaling. Recently reported results unveil a previously unreported mechanical aspect of EphA2 signaling. Specifically, physical restriction of EphA2–EphrinA1 cluster movement by substrate barriers alters the cellular response to EphrinA1 as observed by cytoskeleton reorganization and protease recruitment [44]. In light of EphA2's role in cancer and the known mechanical changes to tissue in cancer, these patterned membrane techniques have helped to reveal a possible molecular mechanism by which tissue mechanics contributes to cancer progression.

4 Techniques for membrane patterning

Patterned supported membrane, by means of controlling spatial organization, chemical functionalization, curvature,

and topography, provides numerous opportunities to probe and control living cells. Various techniques for membrane patterning have been developed since last decade and we present a brief overview of the state of the art.

5 Spatial patterning

Nano/micro-fabricated lines of materials that resist membrane formation (*e.g.* metals, some polymers, and proteins) can serve as passive barriers to molecular lateral mobility in the membrane as discussed above. High-resolution patterns on underlying substrates are achieved by various modern solid-state fabrication techniques, including photolithography, electron-beam lithography, and thin film deposition [9, 26, 39]. Typically, thin chromium lines with 100 nm in width and 5 nm in height are fabricated on the glass substrate. As lipid molecules cannot self-assemble a continuous bilayer membrane over chromium surface, the nanopatterned metal grids on the glass substrate effectively act as barriers for membrane components.

Direct removal of lipid molecules and proteins has also been demonstrated to pattern supported membranes. UV/ozone photolithography, for example, can locally degrade and remove lipids and proteins from supported membranes and the resultant patterns remain intact [55, 58]. High resolution of *in situ* lipid removal can be achieved by multiphoton photolithography using near-infrared Ti:sapphire laser and high NA lens on the microscope [47]. Scanning probe lithography can also be utilized to pattern supported membranes. Using scanning tips of atomic force microscope, lipid molecules can be physically removed from bilayer structures [26, 45]. Patterns generated by direct lipid removal can be preserved by back-filling with blocking proteins, such as bovine serum albumin. Or, when performed on substrates prepatterned with grid lines, depleted corrals can be refilled with another composition of fluid membrane [26].

Lift-off strategies have been implemented to pattern supported membrane as well. A sacrificial layer, such as thin aluminum metal, is prepatterned on the glass substrate. After supported membrane formation, underlying aluminum layer are then etched in mildly basic aqueous solution and lipid molecules over aluminum region are removed [27]. Similarly, prepatterned parylene polymer on the glass substrate can also be lifted off and then create complementarily patterned supported membranes [51].

Other patterning techniques with lower spatial resolutions have also been used in other biological applications. For example, supported membranes in microfluidics channels combine both advantages of small volume in microfluidics and lateral mobility of membrane components and have been greatly utilized as ligand detection

systems *in vitro* [29]. Direct microcontact printing of lipid molecules by plasma-oxidized polydimethylsiloxane (PDMS) stamps have been used to create array patterns of supported membranes [54]. Large scale supported membrane arrays can be printed by microarrayer under humidified conditions and have industrial applications [53].

6 Curvature patterning

Cellular membranes display a variety of morphology with different curvatures. It is believed that membrane geometry could be dynamically regulated in order to provide appropriate environment and to perform various biological processes, including vesicle secretion, motility, and endocytosis. Therefore, controlling membrane curvature is important to study the coupling between membrane deformation and signal transduction [20, 37].

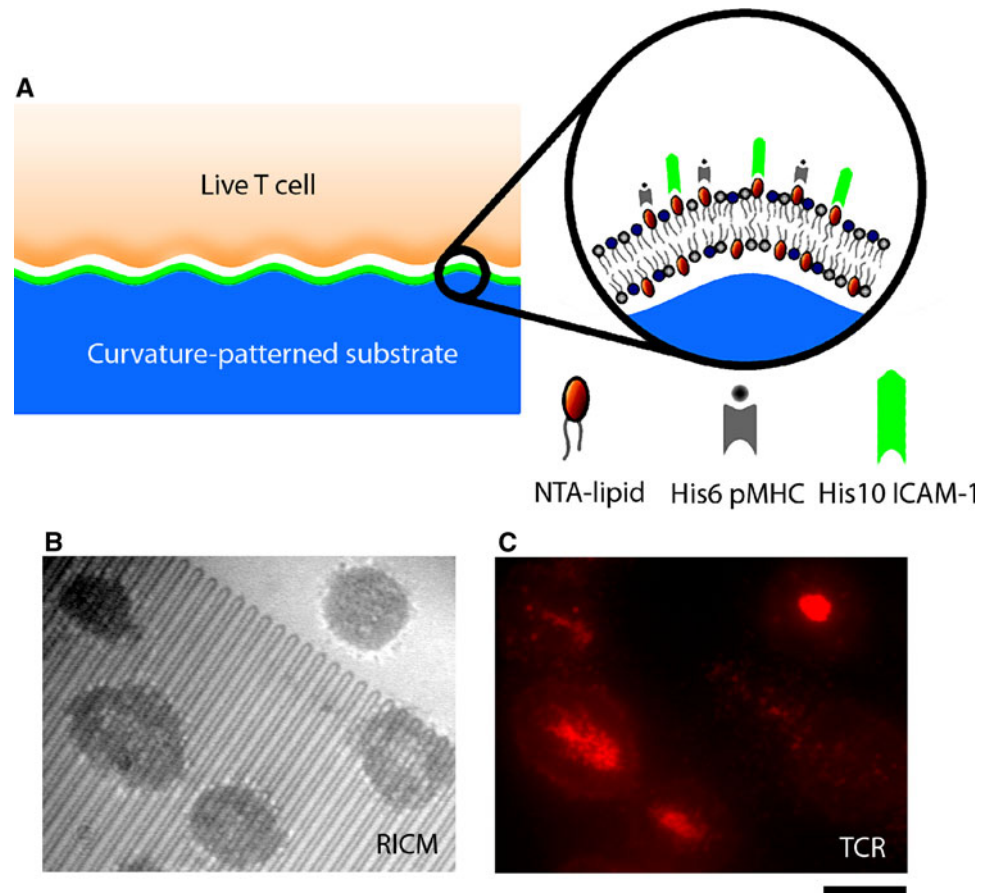
With supported membranes, the modulation of membrane deformation can be accomplished by three-dimensional microfabrication on the underlying substrate [42]. After photolithography, glass substrates are fabricated by combinations of anisotropic and isotropic etching processes. Various substrate curvatures can be achieved by controlling etching processes. Actual surface profile of patterned substrates can then be characterized by atomic force microscopy, for example, to provide high-resolution information. A continuous supported lipid membrane can then deposit on the curvature-patterned substrates by spontaneous fusion of small lipid vesicles.

In the study of cholesterol-dependent phase-separation, controlling curvature in supported membrane has been successfully utilized to spatially organize phase-separated membrane domains [42]. Recently, this membrane curvature modulation technique has been applied to mechanically perturb immunological synapses in live T cells. Initial observation indicates that large scale of protein patterns in the immunological synapse can be altered merely by imposing a defined membrane curvature (Fig. 2). This mechanical perturbation via curvature modulations also changes overall cell morphology. The curvature-patterned hybrid cell junctions surely provide new strategies to study the effect of membrane curvature modulation in cellular signal transduction, such as in cytoskeletal regulations.

7 Biological functionalization

For biological studies, membrane functionalization generally requires the attachment of specific proteins to the lipid bilayer. As supported membranes require aqueous environment to maintain the lipid bilayer structure, a variety of specialized biocompatible techniques have been developed

Fig. 2 Curvature patterning of supported membranes and applications in live T cell immunological synapses. **a** Schematic of imposing membrane curvature onto immunological synapses in live T cells. The modulation of membrane Curvature is accomplished by three-dimensional microfabrication on the underlying substrate. **b, c** Curvature-patterned immunological synapses is visualized by reflection interference contrast microscopy (**b**, RICM) and fluorescence microscopy (**c**). The morphological changes in cell contact area, generally with lower intensity duo to destructive interference in RICM, indicated that membrane curvature plays an important role in cellular responses. Canonical bull's-eye protein pattern of TCR in immunological synapse is distorted merely by imposing membrane deformations. Scale bar 10 μm



to functionalize supported membranes (summarized in Table 1).

Lipid molecules with nitrilotriacetic acid in the head group (NTA lipids) provide stable anchor points using chelation chemistry (Fig. 3). Under neutral pH, proteins which have histidine tags expressed can be chelated with NTA lipids loaded with Ni^{2+} ion [40]. Multiple histidine residues can be sequentially added and ensure stable attachments and preserve proper orientations over supported membranes. In general, 1–5 mol% of 1,2-dioleoyl-sn-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] NTA-DOGS lipids are doped into supported membranes. Ni–NTA lipid–protein linkages have been successfully utilized in various biological researches, such as T cell immunological synapse describe above.

Alternately, lipids with maleimide head group (maleimide lipid) can be doped into supported membranes and allow covalent linkage to thiol group. 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimidomethyl)cyclohexane-carboxamide] (MCC-PE) is commonly added in supported membranes with 1–10 mol% concentration. Molecules with exposed thiol group, such as cysteine residues in proteins, can be covalently linked to maleimide lipid in mild basic conditions (above pH 8) [14].

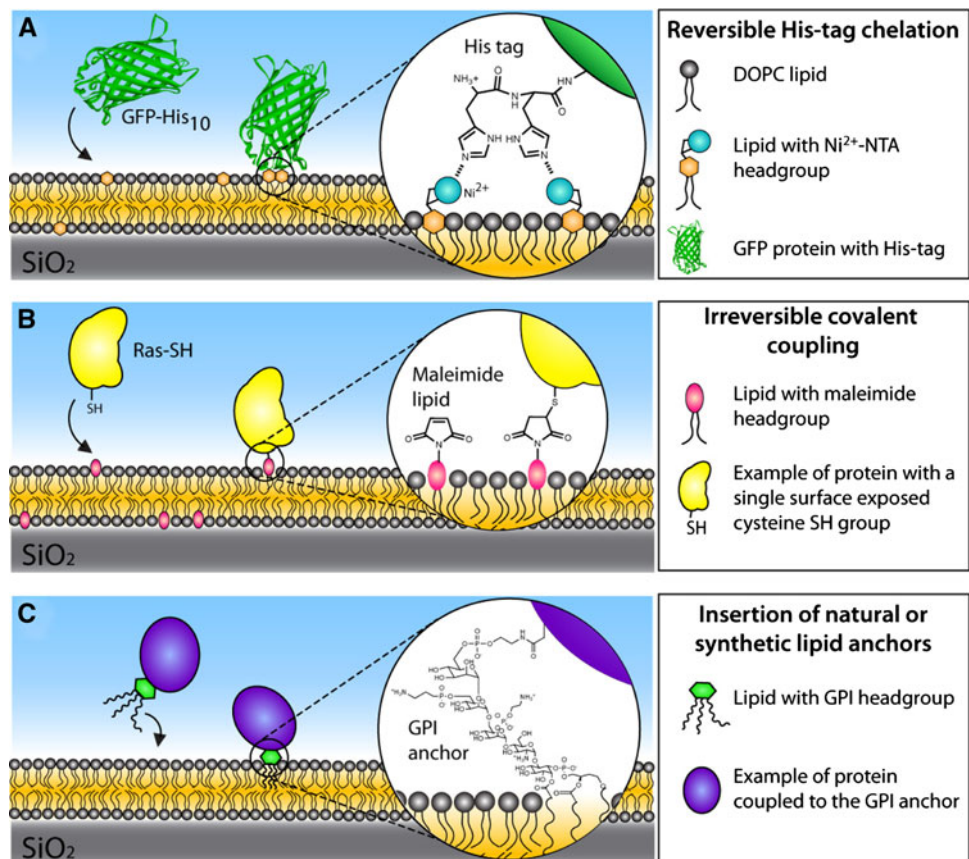
Irreversible and stable attachment of maleimide–thiol lipid–protein linkages have been employed to study kinetics of membrane-dependent activation of Ras signaling systems.

Natural or synthetic glycosylphosphatidylinositol (GPI) linkage is another attachment strategy to supported membranes. Proteins with exposed GPI can be incorporated into supported membranes through the insertion of GPI tails into lipid bilayer structures [18]. This technique was widely used in earlier studies of supported membranes with cells, but has more recently been supplanted by easier linkage systems (especially multiple histidine chelation). One problem with GPI linkages is that they require cell membrane preparations and membrane protein purifications, and thus are much more cumbersome. A more insidious problem with GPI linkages is some evidence for purified GPI-linked proteins to form unnatural aggregations in membranes. Other attachment schemes, such as stable binding between biotin and streptavidin is widely utilized because of its ease and availability. Biotinylated lipid can be doped into supported membranes, and streptavidin can serve as a stable linking bridge between both biotinylated target and lipid molecules [31]. This is a reasonable starting point for membrane functionalization, but it is important to realize

Table 1 Summary of biocompatible techniques to functionalize supported membranes and substrates

Type	Reactive group	Crosslinker	Targeted group	Conditions
Lipid/protein attachment				
Covalent bond	Maleimide group <i>from lipid</i>	N/A	Thiol group <i>from protein or peptide</i>	Unprotected thiol group available in proteins; mild basic condition, pH 8
Multiple chelation	Nickel(II)-NTA <i>from lipid</i>	N/A	Multiple histidines <i>from protein or peptide</i>	Nitrilotriacetic acid (NTA). Multiple histidine residues from proteins to ensure stable chelations
Strong ligation	Biotin analogs <i>from lipid</i>	Streptavidin	Biotin analogs <i>from protein or peptide</i>	Free biotin available in both lipids and proteins
GPI anchoring	N/A	N/A	Glycosylphosphatidylinositol (GPI) tails <i>from protein or peptide</i>	Proteins with exposed GPI can be incorporated into supported membranes through the insertion of GPI tails into lipid bilayer structures
Substrate/protein attachment				
Covalent bond	Thiol-terminated self-assembly monolayer (SAM) <i>on glass</i>	SMCC analogs	Primary amine group <i>from protein or peptide</i>	Heterobifunctional crosslinkers, such as Succinimidyl-4-(<i>N</i> -maleimidomethyl)cyclohexane-1-carboxylate (SMCC), connect thiol groups (pH 6.5–7.5) to primary amine groups (pH 7–9)
Covalent bond	Amino-terminated SAM <i>on glass or gold</i>	SMCC analogs	Thiol group <i>from protein or peptide</i>	
Covalent bond	Carboxyl-terminated SAM <i>on glass or gold</i>	Carbodiimide analogs	Primary amine group <i>from protein or peptide</i>	Carbodiimide analogs react with carboxyl group on the substrate (pH 4.7–6) and then link to primary amine group in target proteins. <i>N</i> -hydroxysulfo-succinimide (Sulfo-NHS) can be added to increase the efficiency
Covalent bond	Maleimide-terminated SAM <i>on glass</i>	N/A	Thiol group <i>from protein or peptide</i>	Unprotected thiol group available in proteins or peptides; mild basic condition, pH 8
Covalent bond	Succinimidyl-terminated SAM <i>on gold</i>	N/A	Primary amine group <i>from protein or peptide</i>	Succinimidyl ester forms covalent linkage with primary amine group (pH 7–9)
Multiple chelation	Nickel(II)-NTA SAM <i>on gold</i>	N/A	Multiple histidines <i>from protein or peptide</i>	Multiple histidine residues from proteins to ensure stable chelations
Multiple chelation	Succinimidyl-terminated SAM <i>on glass</i>	AB-NTA	Multiple histidines <i>from protein or peptide</i>	Succinimidyl ester can be attached to thiol-terminated SAM on glass, and then bind to aminobutyl-NTA (AB-NTA)
Strong ligation	Covalently linked streptavidin <i>on glass or gold</i>	N/A	Biotin analogs <i>from protein or peptide</i>	Streptavidin can be linked to the substrate with various methods above. Biotin can be attached to proteins by native AviTag expression or chemical biotinylation
Strong ligation	Biotin-terminated SAM <i>on glass or gold</i>	Streptavidin	Biotin analogs <i>from protein or peptide</i>	Free biotin available in both SAM and proteins

Fig. 3 Biological functionalization of supported membranes. Various chemical attachment schemes can be utilized to link singling molecules onto supported membranes. **a** Proteins with histidine residues can be chelated with Ni^{2+} loaded NTA-lipids on supported membranes. Multiple exposed histidine residues, such as His6 or His10, can ensure stably attachment within 24 hours. **b** Covalent attachment via maleimide and thiol reactions. Proteins with exposed thiol can be covalently linked to maleimide-lipids. **c** Molecules with natural or synthetic glycosylphosphatidylinositol (GPI) tails can be directly incorporated into supported membranes through the insertion of GPI tails into lipid bilayer structures



that streptavidin introduces a large amount of oligomerization into the system, which could lead to side effects in some situations.

Recently, some strategies to combine immobilized proteins (in controlled patterns) within mobile supported membranes are emerging. Covalent immobilizations are usually achieved by using heterobifunctional crosslinkers [24]. One functional group in crosslinkers selectively reacts with micro- or nano-patterned substrate, such as silanization over glass and self-assemble monolayer over gold. The other reactive group in crosslinkers, such as *N*-hydroxysulfosuccinimide ester, maleimide, and carbodiimide analog, biochemically reacts with target proteins at primary amine, thiol, and carboxyl groups, respectively. Since immobilized molecules inherently serve as diffusion barriers, the combined representation of immobile and mobile components in supported membranes create new possibilities to study membrane signal transduction in cell biology.

8 Polymer-tethered lipid membranes

While lipid membranes on glass substrates create various research opportunities in cell biology, polymer-supported lipid membranes allow ligand integrations even with native transmembrane domains [1, 50]. Phospholipids attached

with polyethylene glycol (PEG) polymer are often utilized as physical cushions to keep transmembrane molecules from nonspecific interactions with supporting substrates. Polymer-supported lipid membranes are proven useful in recent studies of vesicle fusions with molecule complexes of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) in vitro [32]. Currently, spatial-patterning techniques and integration of in vivo cell biological researches using polymer-supported lipid membranes are mostly under development. Nevertheless, polymer-tethered lipid membranes may offer unique solutions to answer certain types of biological questions.

9 Additional applications and future directions

In addition to cell–cell signaling, supported lipid membrane technologies facilitate many biological applications, including lipid membrane arrays for drug discovery [19, 53] and patch clamps for electrophysiology [36]. With advanced optical microscopy techniques, molecular orientations of membrane-associated molecules, such as mucin glycoproteins can be measured with nanometer resolution on supported lipid membranes.[16, 41] Supported membranes with different charged lipid molecules can also be utilized to modulate electrostatic potential on surfaces and

provide alternate strategies for molecule detections [2, 7]. Recently, integration of supported membranes with plasmonic nanocubes effectively forms a label-free biosensor that measures protein binding to lipid membranes [15].

As numerous membrane functionalization techniques have been introduced, orthogonal linking schemes in identical chemical conditions would potentially expand multi-component representations on supported lipid membranes and further mimic the complexity in cellular interfaces. Because of the high selectivity and sensitivity, complementary single-strand DNA hybridization may serve as stable linking schemes between lipid molecules and proteins [5, 6]. Patterned supported membrane technology began to emerge more than a decade ago, and the field has experienced significant growth over the years. Perhaps most importantly, applications of this technology to real problems in cell biology are also developing. Looking forward, we anticipate hand-in-hand growth of both technical capabilities and cell biological applications of patterned membrane systems.

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