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Topography design in model membranes: where biology meets physics

Running title: spatial and curved patterns in artificial lipid membranes

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Abstract

Phospholipid membranes are necessary for the compartmentalisation of chemistries within biological cells and for initiation and propagation of cell signalling. The morphological and chemical complexity of cellular membranes represent a challenge for dissecting the biochemical processes occurring at these interfaces. Therefore, investigations of the biological events occurring at the membrane require suitable models able to reproduce the complexities of this surface. Solid-supported lipid bilayers (SLB) are simplified physical replicas of biological membranes that allow the bottom-up reconstruction of the molecular mechanisms occurring at cellular interfaces. In this brief review we introduce how the properties of SLBs can be tuned to mimic biological membranes, highlighting the engineering approaches for creating spatially resolved patterns of lipid bilayers and supported membranes with curved geometries. Additionally, we present how SLBs have

been employed to reconstitute the biophysical basis of molecular mechanisms involved in intercellular signalling and more recently, membrane trafficking.

Impact Statement

Artificial membranes with complex topography aid the understanding of biological processes where membrane geometry plays a key regulatory role. In this review, we highlight how emerging material and engineering technologies have been employed to create minimal models of cell signalling pathways, *in vitro*. These artificial systems allow life scientists to answer ever more challenging questions in regards to mechanisms in cellular biology. *In vitro* reconstitution of biology is an area that draws on the expertise and collaboration between biophysicists, material scientists and biologists and has recently generated a number of high impact results, some of which are also discussed in this review.

Introduction

Phospholipid membranes allow for compartmentalization of chemical signals within a biological cell. They separate the extracellular milieu from the cytoplasmic interior, defining a cell's boundaries and delimit all the organelles within its cytosol. They also protect the genome from the cytosolic environment and attack from nucleases. Biological membranes are dynamic structures that permit bidirectional transport of cargo, cellular communication and signalling. Therefore, they are more than just a diffusion barrier and participate in cellular signalling, serving as scaffolds and surfaces for protein complexes[1].

The phospholipid bilayer has been known as a functional two-dimensional fluid since the 1970s[2] with the first bilayer structure based on amphiphilic phospholipids presented in 1925[3]. Today, our understanding of molecular processes at the biological membrane has been built upon studies performed on models of the cell membrane. These include structures such as liposomes, giant unilamellar vesicles, monolayers and polymer-supported lipid bilayers[4,5](Figure 1).

The planar lipid bilayers or solid-supported lipid bilayers (SLB) are model systems constructed via self-assembly of lipids on a solid support. They can be engineered to exhibit similar fluid properties of crowded natural cell membranes, displaying lateral diffusion coefficients between 1 $\mu m^2 s^{-1}$ (similar to cell membranes) or slightly faster diffusion rates up to 3-4 $\mu m^2 s^{-1}$, depending on the physical properties of the support interface[6]. The lateral diffusion of lipids in SLBs is also influenced by the liquid-crystalline properties of the bilayer that change with lipid composition[7] and is confined to the plane of the solid support where the lipid leaflets fluidity is sustained by a ~1 nm layer water parting the bilayer from the substrate[8].

SLBs have been utilized to investigate membrane organization[9], protein self-assembly[10], membrane remodelling[11,12] and deduce mechanisms for spatial and temporal initiation of

cell signalling at the plasma membrane[13–15]. The major impact from investigating biochemical processes occurring at the membrane could be in the field of drug development, given that around 50% of drugs target transmembrane proteins[16].

Importantly, SLBs can be designed to be more than just a flat lipid bilayer. Solid support and membrane bilayer morphology can be easily altered to more accurately mimic a system of interest. Specifically, the spatial organization and mechanical deformation of supported membranes can be manipulated by patterning the underlying substrate with modern micro and nanofabrication techniques (Table 1).

In this mini-review, we introduce the technologies to engineer spatially-resolved flat lipid bilayers and SLBs containing patterns of membrane curvature. We discuss how these artificial systems facilitate the study of the biological pathways controlled by the interplay between cellular membranes and membrane-interacting proteins.

Towards building a realistic model of the cell membrane

Cellular membranes exhibit heterogeneity in lipid composition and display a variety of shapes and morphologies, which are necessary for specialization of function. For example, asymmetry in cholesterol lateral composition at the plasma membrane plays a role in the compartmentalisation of protein complexes and in activating and regulating cell signalling via membrane trafficking[17,18]. The function of membrane-bound organelles is linked to their complex morphologies. An exquisite example is the continuous membrane network of the endoplasmic reticulum (ER), with its closely packed membrane sheets. The ER membrane architecture maximizes the surface areas for protein synthesis, whereas the tubules in the peripheral are used for contacting other membrane organelles[19]. Some organelles change morphology progressively to acquire diverse functions, such as endosomes 'maturing' into multivesicular bodies during cargo trafficking to the lysosomes[20].

The earliest model system of a cell membrane was the black lipid membrane, developed by Mueller in the 1960s[21] but it was not very versatile (Figure 1A). Today, liposomes and giant unilamellar vesicles (GUVs) (Figure 1B) offer the possibility to study the role of lipid composition and membrane mechanics in the context of protein-membrane interactions[4]. Generally, GUVs are more appropriate models for studying how a wide range of membrane physical parameters affects their interaction with other molecules. Nonetheless, SLBs offer specific advantages for quantifying kinetic processes at the molecular level since they are amenable to coupling with surface-sensitive analysis techniques, such as surface plasmon resonance (SPR) and quartz microbalance (QCM-D). Low and high-resolution imaging techniques such as atomic force microscopy (AFM), fluorescence recovery after photobleaching (FRAP) and total internal reflection microscopy (TIRF) permit image acquisition of membrane topography, quantification of membrane fluidity and localization of protein assemblies at the supported membrane[22].

There is a choice of methods available to produce SLBs, depending on the extent of surface coverage and the type of solid-support. The direct vesicle fusion[23] method is performed in aqueous buffers and involves liposome interaction, deformation and spontaneous rupture into a single lipid bilayer, coating uniformly the solid substrate, usually silica or mica (Figure 1C). It is the most straightforward and commonly used approach to form SLBs, although it is less suitable to form membranes containing a high proportion of cholesterol, which impacts negatively on the efficiency of vesicle rupture[24]. In the solvent-assisted lipid bilayer method, the bilayer is formed following gradual exchange of a water-miscible organic solvent with an aqueous buffer solution, over lipids deposited onto the solid support[25]. The method is suitable to form bilayers onto a wide range of surfaces (e.g. silicon dioxide and gold[26]) since it does not require an interaction between lipid vesicles and the surface. In the Langmuir-Blodgett (LB) technique[27,28], a lipid monolayer formed at an air-water interface is transferred to a hydrophilic surface. This

technique can be used to produce asymmetric lipid bilayers, which mimic natural cell membranes more closely and are not accessible with the vesicle fusion method (Figure 1D). Recently, spin coating has also been established as a facile method to deposit membranes onto a variety of solid supports[29,30].

A major difference between reconstituted flat lipid bilayers and biological membranes is the lack of the bulk aqueous phase of the cytosol underneath the lipid leaflet, which confers fluidity to cellular membranes. The small hydration layer, spacing the membrane from the solid support, does not accurately mimic the properties of the cellular cytosol. Therefore, in SLBs the solid support and the lipid bilayer are not fully decoupled and the nature of the surface can have major effects on the properties of the membrane. For instance, stable lipid bilayers can form around highly curved substrates, such as 100 nm diameter silica nanoparticles[31], by means of electrostatic interactions between the lipid vesicles and the silica support. Moreover, supported lipid bilayers can adapt to changes induced by substrate plasticity[32,33], a behaviour that can be exploited to control the mechanical properties of the membrane.

In biological membranes, cytoskeletal scaffolds disengage the lipid leaflets from the bulk phase of the cytosol. Synthetic materials that exhibits mechanical properties similar to those of the cytoskeleton, can be sandwiched between the bilayer and the solid-support. For example, polymer brushes[34] or generic polymer nanofibers[35] can be used to recapitulate the function of actin networks and provide the reconstituted lipid bilayer with fluidity properties close to those observed in biological membranes. These polymer-supported SLBs can also be spatially patterned and incorporate proteins of interest[36,37] (Figure 1E). Conversely, SLBs have also been used also to reconstitute the assembly of cytoskeletal proteins, such as the polymerization of an actin scaffold on an SLB formed by vesicles containing ponticulin, an initiator of actin self-assembly[38].

Engineering of spatially patterned bilayers to investigate cell signalling

SLBs can be engineered as spatially-organized patterns of lipid bilayers with specific compositions. Several methods for spatial patterning of membranes have been developed based on approaches where either the membrane is locally confined by the substrate topography (e.g. microcontact printing)[39–41] or alternatively, parts of the membrane are removed after lipid bilayer deposition (e.g. photolithographic techniques)[42–45]. Therefore, solid-supports can be engineered to introduce *ad-hoc* morphological features to study the biological process of interest. An overview of methods for patterning solid-supports is presented in Table 1 and summarized below.

Micro-contact printing is a fairly versatile approach to form patterned membranes. It is based on a form of soft lithography as it utilizes soft elastomers, typically polydimethylsiloxane (PDMS)[39]. The PDMS surface is patterned and used as a contact stamp to transfer a thin layer of material onto a substrate. Its main limitations include stamp deformation or stretching and compressing of the stamp, introducing unwanted features into the pattern. During transfer, the ink can laterally spread into unwanted regions not replicating the desired pattern[46]. Polymer 'lift-off' techniques are a form of microcontact printing where a thin layer of a sacrificial material (aluminium or a polymer) is patterned onto glass. Once a SLB has formed, the material is etched away removing lipids in the patterned regions[47–49] (Figure 2A).

Currently, the techniques that can fabricate membrane arrays with the highest resolution are electron beam[50] and scanning probe lithography[42], which can reproduce features down to 10 nm in size (Table 1). Scanning probe lithography uses an AFM tip to pattern substrates, meaning that features with size beyond the diffraction limit, which limits the achievable resolution in conventional lithography, are accessible via this technique. Other

types of scanning probe lithography either involve removal of material from the surface of the substrate (thermal scanning probe lithography)[43] or use diffusion at the tip of the scanning probe to coat patterns onto substrates, such as alkanethiol arrays on gold substrates (dip-pen lithography)[44,51]. Patterned bilayers can be formed by removal of lipids from a membrane through the use of UV/ozone photolithography[52,53] or using atomic force microscopy[54]. Multi-photon lithography exploits the chemical changes occurring in substrates that can simultaneously absorb two or more photons, such as hydrogels, natural polymers and even proteins[55]. The critical size of the features generated with this technique can be as small as 100 nm, since the chemical transformations occur in a region with a volume of few attolitres[56].

Patterned SLBs provide an advanced platform for the investigation of complex biological processes at the molecular level. For instance, they can be used to study molecular processes occurring during endocytosis[57], cell-cell interactions[58] and communication[59,60]. Lipid membranes can be spatially-organized with micrometre size diffusion barriers and employed to study molecular processes occurring within confined geometries, such as the molecular details of T-cell activation[61]. Similarly, patterned arrays of lipid bilayers[62] can be used as sensor arrays for cell adhesion[13,63]. Physical barriers for lipid diffusion can be made using masks created from materials that prevent membrane formation (e.g. metals, some polymers). These barriers are usually taller than the average height of a lipid bilayer (~ 4 nm) but recently, it has been shown that graphene[64] offers the possibility to create diffusion barriers as thin as one-atom.

The role of transmembrane receptor spatial organization in intercellular signalling has been extensively studied using patterned SLBs[65]. At the plasma membrane, internal and external inputs can alter the spatial organization of cell surface receptors. For instance, intercellular signalling requires close contact between transmembrane receptors to allow direct communication between neighbouring cells. Reproducing *in vitro* an intercellular

junction is a complex task but a combination of solid-state nanolithography and supported lipid bilayer techniques can reveal the interplay between spatial organization and transmembrane receptor activity[66]. For example, Green et al.[67] used electron beam lithography to create physical barriers to lipid bilayer lateral mobility creating membrane corrals containing the tyrosine kinase receptor Ephrin A1 (EphA1). This system was used to guide breast cancer cells to the EphA1-patterned membranes via the interaction with the cognate receptor Ephrin A2 (EphA2), thereby creating a semi-synthetic junction. This spatial setup of ephrin receptors revealed the relevant molecules involved in the endocytosis of EphA2, an important step in the regulation of the EphA2 receptor signalling pathway[67].

Similar experimental approaches have been applied to the investigation of events involved in the mechanical regulation of cell surface proteins. For instance, patterned SLBs have been employed to study the relationship between the organization of Epidermal Growth Factor Receptor (EGFR) and its phosphorylation levels[68] and for the understanding of Ras signalling at a single-molecule level[69].

Engineering curved membranes to understand the mechanisms of membrane remodelling

Membrane geometry and cellular signalling are intimately connected[70], particularly in biological processes where macromolecular assembly at the membrane is regulated by curvature[1,71,72]. Membrane shape and lipid composition are features of membrane organelles, which are not only evolutionarily conserved but are also finely controlled to maintain spatial segregation of molecules during membrane trafficking[73,74]. Interestingly, over a specific threshold (~0.8 μm^{-1}), curvature appears to regulate the spatial organization of lipid phases including cholesterol[75], and also the segregation of lipids such

as hexadecanoic acid[76] and cardiolipin[77]. Importantly, local membrane curvature also controls the segregation of curvature-sensing proteins[78](Figure 2B). For instance, lipidated proteins appear to be selectively recruited by regions of specific membrane curvature[79]. The correlation between substrate curvature and lipid organization underlines the feedback mechanisms that regulate the interaction between the membrane and protein assemblies active at the membrane[80]. Therefore, methods to introduce regions of curvature in model membranes are highly desirable to study biological mechanisms where lipid sorting and membrane deformation are crucial mechanistic steps in recruiting protein complexes to membranes[81], such as membrane trafficking.

Curved supported membranes are formed via direct fusion of lipid vesicles onto the pre-patterned solid-support. Localized regions of curvatures can be introduced in lipid bilayers by means of grooves, edges or other non-flat features at the surface of the solid-support. For example, patterns of curvature in SLBs can be produced by manufacturing, with a focussed-ion beam, cylindrical concavities on a chromium-coated coverslip onto which the lipid bilayers are assembled[82]. Alternatively, nanopits[83] and nanoparticles[84] can be used to guide the formation of regions of local curvature in lipid bilayers. It is worth nothing that there is a physical limit to the curvature achievable for a phospholipid membrane on a solid support. This was exemplified by the study of Roiter et al. [85] which explored the behaviour of membranes deposited on surfaces decorated with nanoparticles. They observed that lipid bilayers form continuously on silica nanoparticles smaller than 1.2 nm or larger than 22 nm, whilst pore formation in the membrane around the particle was likely within this size range.

Non-flat membranes facilitate the investigation of processes in which an asymmetric segregation of lipids occurs laterally or between the leaflets of the membrane at very high curvatures (e.g. tens of nm)[86]. Coupling between lipid structures and curvature preference is a consequence of the interactions between different lipid shapes, which prefer to

arrange as locally curved bilayers [75,87]. Lipid sorting can be a key signal to drive transmembrane protein clustering[88] and cell signalling[89,90]. For instance, lysophosphatidic acid displays an inverted cone shape that confers its tendency to populate regions of membranes with positive curvature[87,91,92] (Figure 2C).

Complex membrane topographies containing curves and ridges[93] can enable the investigation of how protein complexes remodel phospholipid membranes. Membrane remodelling proteins (and assemblies thereof) display curved structures, which are used for sensing curvature and for inducing further membrane bending[1,81,94]. Special types of SLBs have been recently developed to specifically follow membrane remodelling reactions. The SUPported bilayers with Excess membrane Reservoir (SUPER)[95,96] are supported bilayers, assembled on micron-scale silica beads in a manner that results in generating a loosely fitted membrane on the solid support (Figure 2D). The excess membrane allows for membrane remodelling processes to occur in the presence of the solid support and be monitored by fluorescence and electron microscopy. These supported membranes have been successfully used to reveal the physical basis of membrane remodelling promoted by protein crowding[97] or performed by endocytic[98–101] and autophagic proteins[102]. Similarly, the supported membrane tubes (SMrT)[103] are synthetic membranes mimicking membrane tethers pulled by proteins[104,105] that can be used to study the impact of local curvature on protein-membrane interactions[101].

A unique membrane remodelling protein complex has recently received attention in the membrane biophysics community, the Endosomal Sorting Complex Required for Transport (ESCRT). ESCRT function is vital for key cellular processes such as multivesicular body biogenesis (MVB), cytokinesis, neuron pruning, plasma and nuclear membrane repair and viral budding[106,107] (Figure 3A). In all these processes, the topology of membrane deformation by ESCRT is the same: the budding occurs away from the cytosol[108], in contrast, for example, to bud formation by dynamin in endocytosis[101,109].

Given its unique activity, the biophysics and biochemistry of budding and scission reactions by the ESCRT complex have been the focus of intense investigations. The assembly of ESCRT proteins into a functional complex have been reconstituted with GUVs[110,111] and onto SLBs to dissect the biophysical basis of membrane remodelling in the context of multivesicular body formation. The main scission machinery of ESCRT, namely ESCRT-III, has been observed to assemble into concentric circle-like structures on lipid bilayers[112,113] (Figure 3B). These studies suggested a potential mechanism for membrane deformation that involve flat 'spirals' of ESCRT-III acting like springs. ESCRT-III spirals undertake lateral compression on the membrane and incorporate elastic energy, which leads to membrane deformation upon energy release (Figure 3C)[113]. In another study, SLBs were used to decipher how the growth of ESCRT-III circular structures is self-regulated and how ESCRT-mediated membrane remodelling is coupled to ATP hydrolysis[114].

ESCRT proteins assemble and stabilize the neck of a budding membrane; therefore, membrane curvature plays a role in protein recruitment and assembly at these locations[106]. Lee et al. [82] created a SLB with features resembling membrane bud necks by assembling a lipid bilayer on a substrate with 100 nm deep invaginations, presenting regions of negative curvature (Figure 3D). TIRF microscopy was used to monitor the assembly of the human ESCRT-III complex at the membrane, which revealed clustering of the proteins specifically at the invaginations in the membrane, demonstrating ESCRT activity at membrane bud necks[82] (Figure 3E).

Perspective and Conclusions

We have presented a brief overview of the solid supported lipid bilayer technology and reviewed the fabrication of spatial and curved patterns in the context of the *in vitro* reconstitutions of biological processes at the membrane.

Flat synthetic membranes are a powerful tool to investigate how the biophysics of lipid-lipid interactions contributes to the assembly of complex biological structures[115–117]. Advances in the engineering of structural features in solid supports is allowing the generation of ever more complex spatial or curvature patterned bilayers, thus creating more realistic mimics of cellular membranes. SLBs with rough surfaces facilitate the systematic dissection of the interplay between curvature and lipid sorting and crucially, how these physical properties are coupled and controlled in a complex biological process. Lipid phases can be coupled with nanometre-scale curvature patterns[118], providing a strategy for organizing a controlled segregation of biomolecules and lipids.

A yet unmet challenge in the SLB field is the *in vitro* reconstruction of more complex biological membranes, such as the nuclear envelope. The nuclear envelope is a double membrane[119] in which transmembrane proteins couple the inner membrane to chromatin and the outer membrane relay signals from the cytoskeleton[120]. Such an *in vitro* model would allow the study of the molecular basis of epigenetic mechanisms and nuclear envelope membrane repair in physiological and diseased contexts[121]. Whilst SLBs comprised of multiple bilayers have been recently developed[48,122], we are still far from being able to design a fit-for-purpose model of this structure. Therefore, it appears that the field is ripe for moving towards increasing complexity with the destination of yet more complex models allowing us to interrogate biological mechanisms and/or reconstruct cell biology from the bottom-up.

Author contributions

All authors contributed equally to the writing and editing of this manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Tables

Table 1. Outline of the techniques for patterning SLBs, their resolutions and disadvantages/advantages.

Figures

Figure 1. In vitro approaches used as models of the biological membrane. (A) Black lipid membranes are formed when dried down lipids are hydrated with an aqueous medium over an aperture between two aqueous phases. (B) Liposomes (50 nm) or giant uni-lamellar vesicles (100 µm) that self-assemble into these structures when dried down lipids are hydrated in an aqueous medium. (C) In the vesicle fusion method, liposomes are incubated onto a hydrophilic surface where they fuse, deform and spontaneously rupture to form a uniform, continuous, fluid lipid bilayer (D) Langmuir-Blodgett technique: Lipid monolayers are formed at the air-water interface when lipids are added to an aqueous medium. This monolayer is transferred onto a hydrophilic solid support, this transferal process is done twice to form a lipid bilayer. The green arrow indicates the direction the substrate (grey) is moving to pick up another monolayer of lipids (E) Polymer supported/tethered lipid bilayers are formed on top of a polymer cushion hydrogel and also tethering units such as nickel NTA for anchoring of proteins.

Figure 2: Examples of spatial and curved patterns of lipid bilayers on solid support. (A) High quality, fully mobile SLBs forming well-defined patterned arrays of phase-segregating DOPC:DSPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)) SLBs through the polymer stencil lift-off technique. DHPE-LR (red) (Lissamine rhodamine-labeled 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine) partitions preferentially with DOPC (Lα phase), rendering the

DSPC (Lβ phase) domains dark. The insets are magnified images of the small circular and square patterns. Image reproduced from reference 47 with copyright permission. (B) Curvature-sensing proteins incubated on fluid wavy membranes and visualized via confocal fluorescence microscopy. Partitioning of ENTH-GFP, N-BAR-Alexa Fluor 488 and BIN1 N-BAR-Alexa Fluor 488 into positive-curvature regions. Image reproduced from reference 78 with copyright permission. (C) Spontaneous lipid sorting occurs for specific lipid mixtures. For instance, lysophosphatidic acid (LPA) inverted conical shape matches best positive curvature regions when in combinations with cylindrical lipids such as DOPC and both tend to distribute in the outer membrane leaflet. If present, DOPE (DOPE is 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) prefers to occupy the inner membrane leaflet (adapted from reference 87). (D) SUPER templates are formed by exploiting the swelling of membranes in high ionic strength buffers. Fusion of liposomes on size silica nanoparticles (~5 nm) in high ionic strength solution, generates bilayers with excess membranes (adapted from reference 96).

Figure 3. **Membrane remodelling by the ESCRT complex**. (A) Brief overview of some of the cellular functions of the ESCRT complex in mammalian cells. ESCRT-III remodel membranes away from the cytosol. The core remodelling machinery (ESCRT-III) is indicated as a blue spiral. ESCRT-III organises as circular filaments to stabilise the neck of membranes buds. (B) Atomic force microscopy topographic image of the centre of a Snf7 patch assembled on GUV membrane. Image reproduced from reference 113 with copyright permission. (C) The spiral structure acts as a spring collecting elastic energy which upon release buckles the membrane. (D) 3D surface plot of the atomic force microscopy profile showing lipid bilayer formation on top of the 100-nm-deep invaginated template for negative curvature, which approximates the shape of a nascent HIV-1 bud produced by focused ion beam. Image reproduced from reference 82 with copyright permission. (E) Total internal reflection fluorescence (TIRF) image after 20-minute

incubation with 400 nM of the human ESCRT-III core subunit CHMP4B-Atto488 (DiD

fluorescein used as blue marker for lipids). Image reproduced from reference 82 with

copyright permission.)

Table 1.

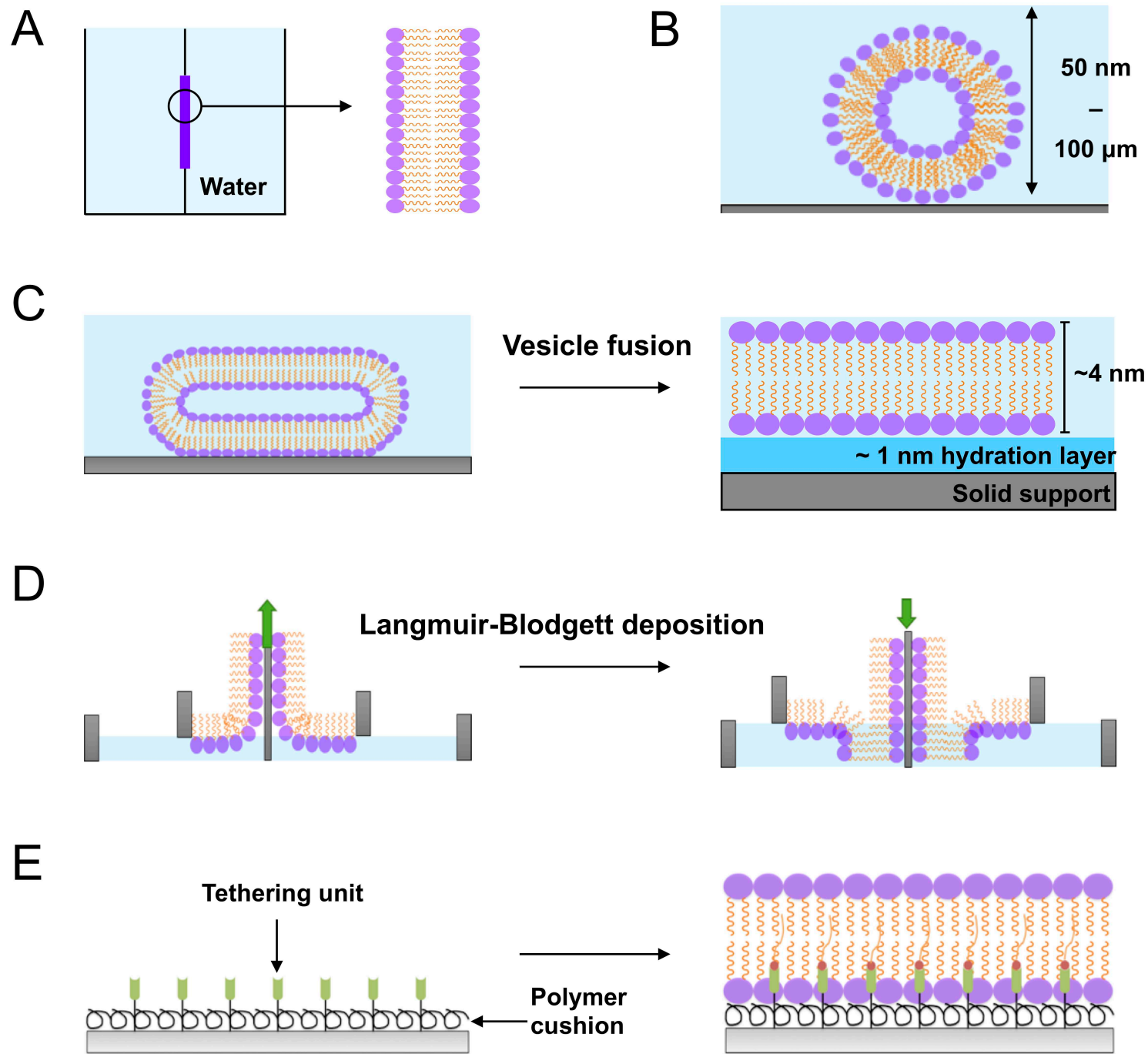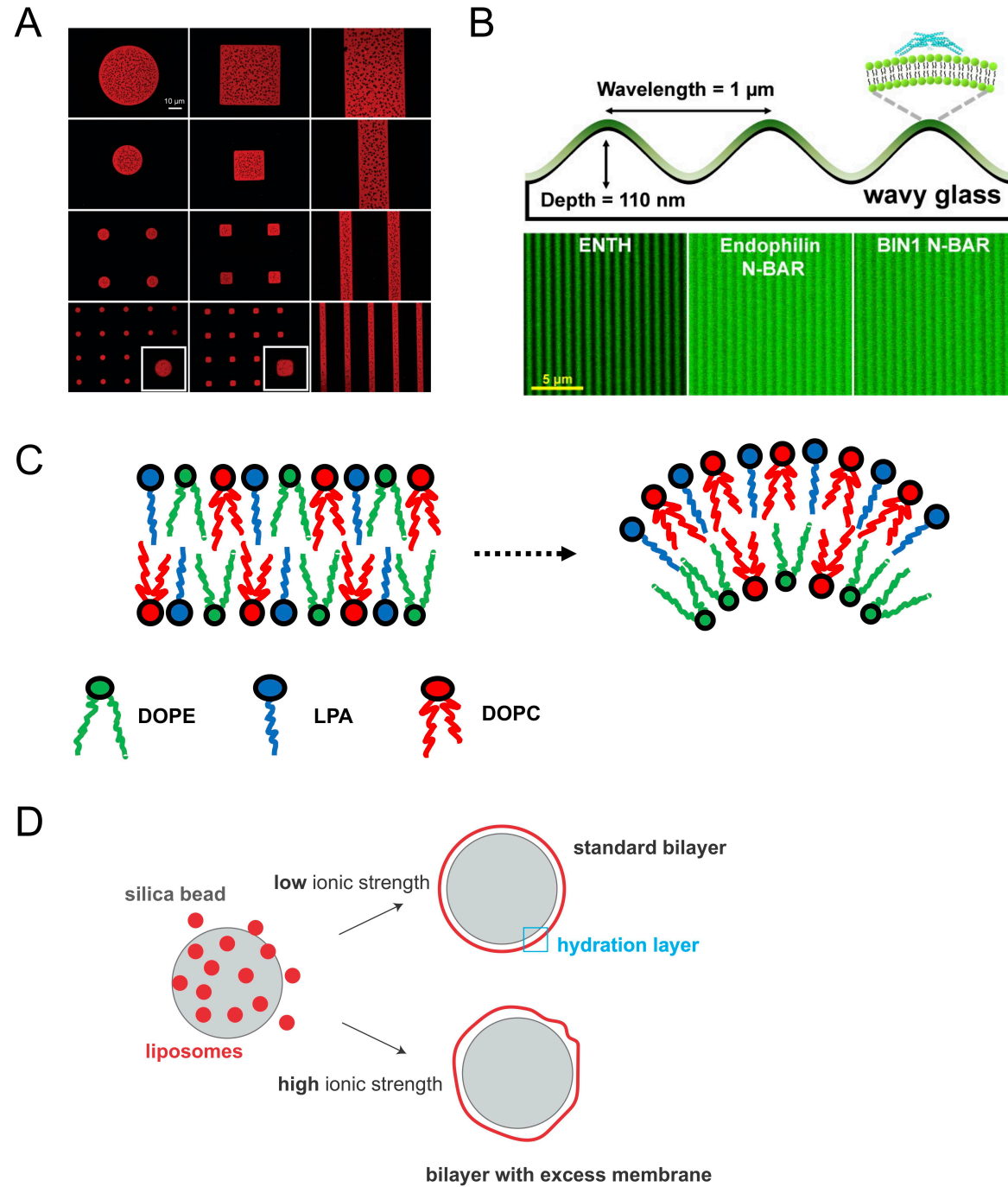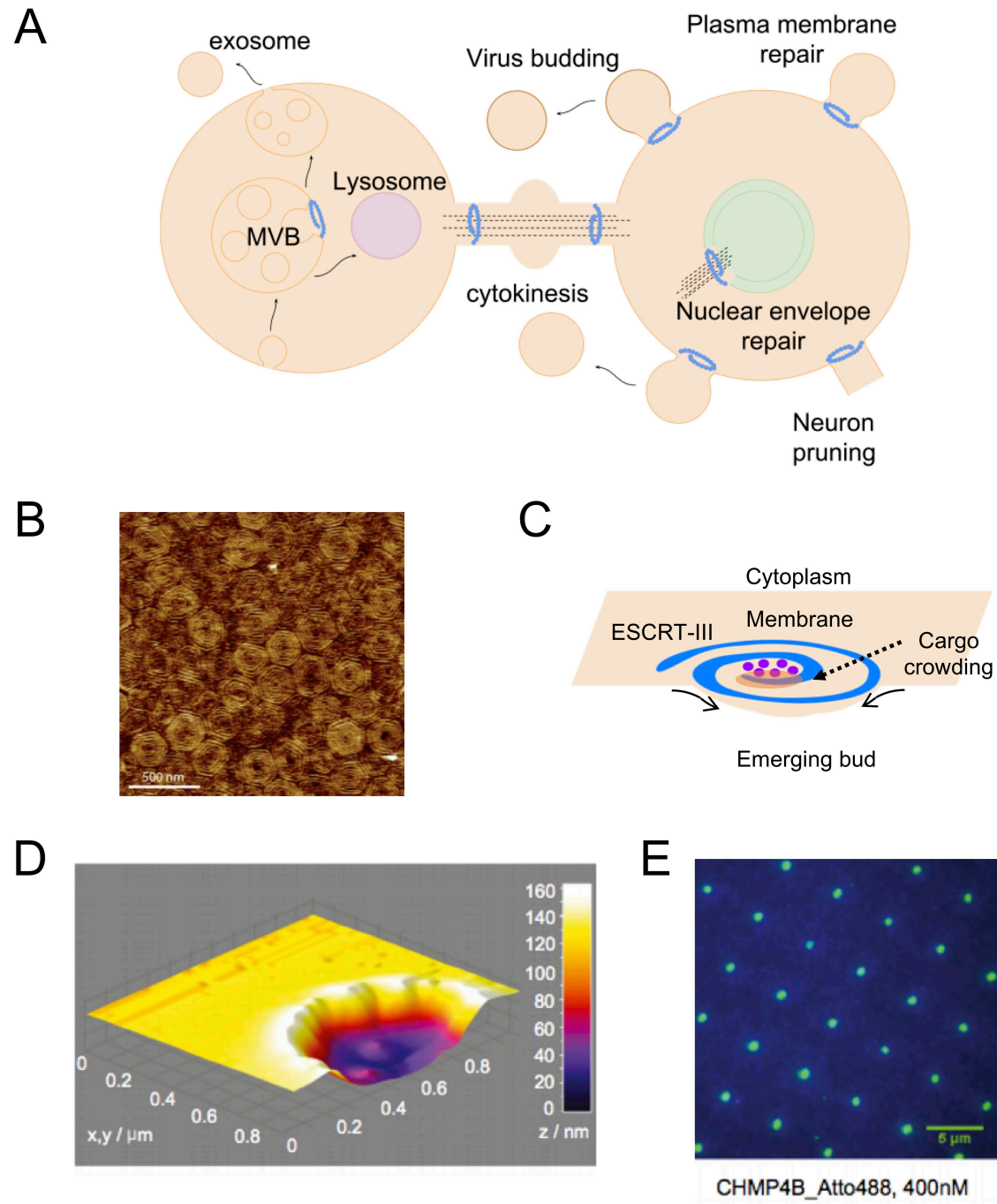| Technique	Patterning process	Lateral Resolution	Advantages	Disadvantages	References
Micro-contact printing	Form of soft lithography that fabricates structures using elastomeric stamps, molds and conformable photomasks.	500 nm	• No photo-reactive surfaced needed • Resolution can reach tens of nm	• Stamp deformation • Shrinking/swelling of the stamp • Ink diffusion	40,46
Photo-lithography	Uses light to transfer a pattern from a photo-mask to a light sensitive chemical (photoresist), on a substrate.	<100 nm	• Exact control of pattern shape and size • Patterning over the entire surface	• Requires extremely clean operating conditions • Not effective for creating non-flat shapes	123
Scanning probe lithography	Set of nano-lithographic methods to pattern materials using scanning probes.	10 nm	• High resolution as the process bypasses the diffraction limit.	• Slower process in comparison to photolithography.	42,54,124
Electron beam lithography	A beam of electrons is scanned over a surface covered with an electron-sensitive resist, removing regions of exposed or non-exposed resist to draw a pattern.	<10 nm	• High resolution • Direct writing of custom patterns.	• Beam drift and backscattering electrons can cause physical defects.	50,125
Multi-photon lithography (direct laser writing)	Structuring is achieved by direct fabrication using near infrared femtosecond lasers to induce polymerization at the focal point.	100 nm	• Does not require a photo-mask or multiple processing steps. • 3D Fabrication	• Short working distance required with objective lens limits the height of the fabricated structures.	45,55,56

Figure 1

Figure 2

A

exosome

Virus budding

Plasma membrane repair

Lysosome

MVB

cytokinesis

Nuclear envelope repair

Neuron pruning

B

500 nm

C

Cytoplasm

Membrane

ESCRT-III

Cargo crowding

Emerging bud

D

E

CHMP4B_Atto488, 400nM

5 µm

Figure 3