Native supported membranes: Creation of two-dimensional cell membranes on polymer supports (Review)

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I. INTRODUCTION

In nature, membranes serve as fundamental platforms for many important biological processes, such as (i) hormone transduction and amplification through generation of second messengers, (ii) biosynthesis by membrane associated ribosomes at the endoplasmic reticulum, (iii) control of cell adhesion via protein-protein recognition, and (iv) lateral segregation of cell-surface receptors and the associated reorganization of the membrane-coupled cytoskeleton. Phospholipid bilayers deposited onto solid substrates (called solid-supported membranes) have been the simplest and most commonly used experimental model systems that allow us to gain insights into structure-function relationships in biological membranes.[1–3] Supported membranes retain the intrinsic "fluid" nature to self-heal local defects and achieve an excellent mechanical stability. These properties offer distinct advantages over either freestanding black lipid membranes or spherical lipid vesicle suspensions: they can be subjected to various surface-sensitive physical characterization techniques such as ATR-FTIR,[4] surface plasmon resonance,[5] quartz crystal micro balance,[6] and neutron reflectivity.[7]

However, in spite of great scientific achievements through solid-supported membranes, such systems have some fundamental drawbacks. Solid-supported membranes are confined at a deep potential minimum governed by van der Waals interactions and stay in close proximity of solid substrates. The water reservoir between the membrane and the underlying solid substrate, which is typically 5–20 Å,[7–9] is not thick enough to prevent proteins coming into direct contact with the bare substrate. In fact, this can cause a serious problem in dealing with transmembrane proteins, such as cell adhesion receptors, whose functional extracellular domains can extend to several tens of nanometers. In fact, the spreading of proteoliposomes doped with human platelet integrin $\alpha_{IIb}\beta_3$ and ATP synthase on quartz or glass substrates results in inhomogeneous patches of "pinned" proteins [Fig. 1(a)].[10–12]

Such problems can be overcome by separating the membrane from the solid substrate using soft polymeric spacer layers, whose thickness is less than 100 nm. Here, the membranes are separated from underlying solids via (i) polymer cushions[13,14] or (ii) polymer/oligomer tethers.[15–19] This minireview focuses on the former systems, which can be used for deposition of various natural biological membranes (Fig. 1).

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II. POLYMER SUPPORTS AS ARTIFICIAL EXTRACELLULAR MATRIX AND CYTOSKELETONS

One of the most important criteria when choosing an appropriate polymer support is to achieve thermodynamically and mechanically stable membranes. In fact, the deposition of cell membranes on polymer supports can be generalized as the wetting of complex fluids. For example, to achieve a stable layered supramolecular architecture that mimics one-half of a cell-cell contact (Scheme 1), one must carefully adjust the interactions at each interface; namely, the interaction at the surface/polymer interface [generally described as surface tension σ_{SP} (N/m)], at the polymer/membrane interface (σ_{PM}), and at the membrane/water interface (σ_{MW}). From a thermodynamic viewpoint, such stratified structures are only stable if the presence of an "additional" layer (i.e., a cell membrane) results in a gain in the total surface free energy. Within the framework of physics of wetting, this can be referred to as a complete wetting scenario, which can be characterized with a positive spreading coefficient $S=\sigma_{SW}-(\sigma_{SP}+\sigma_{PM}+\sigma_{MW})\geqslant 0$.[20,21] Moreover, the interaction between the membrane and the surface needs to be repulsive. In fact, if the net force acting per unit area (disjoining pressure) is negative, continuous thinning of the interlayer results in the film collapse, i.e., dewetting, at the pinning centers.[21,22] Thus, polymer cushions mimic the generic roles of the extracellular matrix and the cell-surface glycocalyx, which in nature maintain within themselves a relatively high osmotic pressure that keeps finite distances (of typically 10–100 nm) at the cell-cell and cell-tissue contacts. Through this mechanism, nonspecific contacts due to van der Waals attraction, which are effective over distances up to about 3 nm, are effectively suppressed.

The presence of hydrated polymer cushions underneath the supported membranes reduces the frictional coupling between membrane-incorporated proteins and the substrate surface [Fig. 1(b)]. If one generalizes the diffusion of transmembrane proteins in a polymer-supported membrane as the diffusion of cylindrical particles in a two-dimensional medium that is separated from a wall via a viscous medium of defined thickness d and viscosity η [Fig. 1(c)], then the drag

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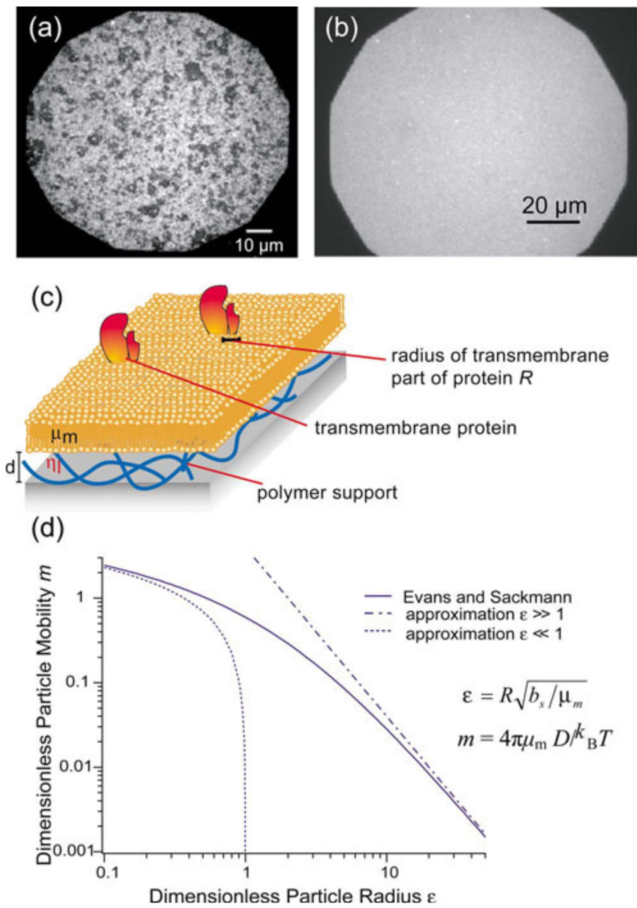


FIG. 1. (a) Fluorescence image of a solid-supported membrane doped with fluorescently labeled human platelet integrin $\alpha_{IIb}\beta_3$. (b) The same integrin-doped membrane deposited on a cellulose cushion (dry thickness: 5 nm). (c) Schematic illustration of the lateral diffusion of transmembrane proteins in supported membranes. (d) Relationship between dimensionless particle mobility m plotted vs dimensionless particle radius ε (Ref. 44).

coefficient in the Einstein equation $f=k_BT/D$ can be expressed as a function of the dimensionless particle radius of the diffusant ε,

$$f=4\pi\mu_m\left(\frac{1}{4}\varepsilon^2+\frac{\varepsilon K_1(\varepsilon)}{K_0(\varepsilon)}\right), \quad (1)$$

k_B is the Boltzmann constant, μ_m is the two-dimensional membrane viscosity, and K_0 and K_1 are modified zero and first orders Bessel functions of the second kind. The dimensionless particle radius ε is defined as a function of the radius of the transmembrane part of a protein R and frictional coefficient $b_s=\eta_1/d$,

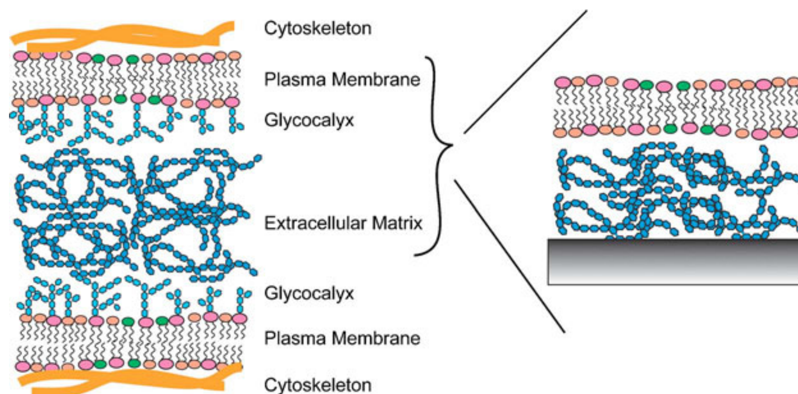
$$\varepsilon = R\sqrt{b_s/\mu_m}, \quad (2)$$

ε can analytically be obtained from the dimensionless particle mobility $m=4\pi\mu_m D/k_BT$. [Fig. 1(d)]. This means the design of polymer supports with defined d and η enables one to control the frictional drag exerted to transmembrane proteins in a quantitative manner.

The advantage of using polymer supports can also be seen when one studies the biological functions of incorporated cell receptors such as integrin in a quantitative manner. When the interaction between the integrin-doped polymer-supported membranes and giant vesicles exposing integrin-specific ligands was monitored by microinterferometry, the adhesion free energy was found to be three to ten times higher than the adhesion energy obtained with solid-supported membranes containing the same amount of integrin receptors.[10,11] The binding energy between integrin and the ligand calculated from the lateral protein density was comparable to the one calculated from the dissociation constant, suggesting that the integrins maintain their native adhesion function in the polymer-supported membranes.

III. PREPARATION AND EXTRACTION OF NATIVE MEMBRANES

Native cell membranes can be extracted from various sources such as bacteria[23] human erythrocytes,[24,25] or vertebrate tissues,[26] according to the protocols including density gradient centrifugation to separate intracellular membrane systems or organelles.



SCHEME 1. Schematic illustration of a contact between neighboring cells, mediated via hydrated polymer layers, such as glycocalyx and extracellular matrix (left). A polymer-supported membrane (right) can be used as a well defined artificial model system.

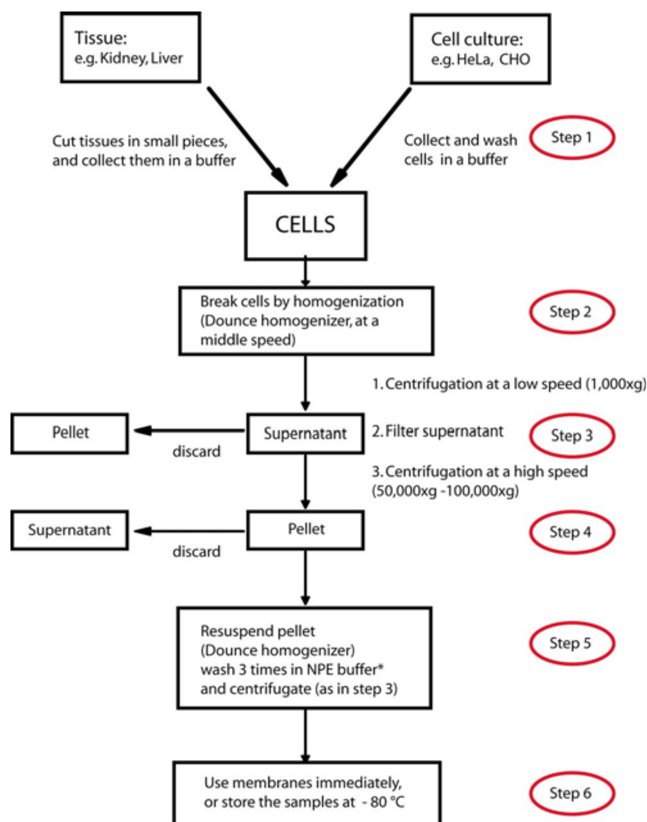


FIG. 2. General overview of the preparation of native membranes from tissues and cell cultures. NPE buffer (*p*H 7.4): 10 mM NaH_2PO_4, 1 mM EDTA and 400 mM NaCl.

Figure 2 represents a general overview of isolation and purification of cell membranes from single cultured cells or tissues. First, tissue cells (muscle, liver, kidney) are mechanically ruptured using a warring blender, whereas single cells are collected from the culture after trypsination and washed three times in buffer (step 1). Afterwards, the cells are resuspended in a special lysis buffer that contains protease inhibitors such as phenylmethylsulfonylfluorid, aprotinin, leupetin, pepstatin A, and/or ethylenediaminetetraacetic acid (EDTA) and carefully homogenized. Cell lysis and all the following preparative steps should be performed at 0 °C (on ice) in order to minimize the protease activity. It is notable that homogenization of the membrane (step 2) is one of the most crucial steps, since too extensive homogenization at a high speed may induce the rupture of cell nuclei that eventually results in the contamination of the sample with nucleic acids. Cell nuclei, cell debris, and nonruptured cells are removed by a short, gentle centrifugation (at $\sim 1000g$, step 3). The pellet is discarded, while the supernatant is filtered through eight layers of gauze. The filtered supernatant is subjected to an ultracentrifugation at $50\,000-100\,000g$ to sediment the homogenized, crude membrane extracts (step 4). In the next step, the supernatant is discarded, and the remaining pellet is carefully homogenized and washed three times in NPE buffer (step 5). The resulting crude membrane fraction can be used immediately, or the samples can be stored at −80 °C after freezing in liquid N_2.

IV. FABRICATION AND CHARACTERIZATION OF TWO-DIMENSIONAL CELL MEMBRANES

Nature stringently controls the orientation and the population of transmembrane proteins. It can even dynamically adjust plasma membrane composition in response to events or stimuli, as evidenced by transfection of human erythrocyte leading to an increase in the fraction of a particular protein class (band III proteins). Replicating this degree of control using supported membranes is difficult. Transmembrane proteins are usually first stabilized in surfactant micelles and then incorporated into lipid vesicles. Afterwards, proteoliposomes are spread to create supported membranes. However, it is practically difficult to incorporate complex and concentrated protein mixtures into vesicles to produce supported membranes that mimic more closely the complexity of natural membrane compositions.

One breakthrough to overcome this problem is to spread native cells or microsomes onto planar substrates instead of artificial vesicles. Here, polymer interlayers are more advantageous over naked solid substrates, since they can finely tune the cell-surface interactions. In fact, adult animal cells, which express negatively charged sialic acid residues, do not adhere on bare glass or quartz substrates due to the electrostatic repulsion. The first successful deposition of native membranes on planar supports was reported for human erythrocyte “ghosts” (red blood cells after removal of their cytoplasm) spread over cellulose cushions. After incubation of ghost cells for 60 min, the orientation of the erythrocyte membrane is identified with two immune-fluorescence labels: (1) the extracellular part of glycophorin can be labeled with a first monoclonal antibody (mouse IgG) and a second polyclonal antibody (goat antimouse IgG) with tetramethylrhodamine isothiocyanate (TRITC) (outside label), while (2) the cytoplasmic domain of band 3 can be recognized with a first monoclonal antibody (mouse IgG) and a second TRITC-labeled polyclonal goat antimouse IgG antibody (inside label). Immune-fluorescence labeling proved that polymer-supported human erythrocyte membranes are almost free from any local defect and expose the cytoplasmic domain to the bulk buffer [Figs. 3(a) and 3(b)].[27] Continuous coverage of the surface with cell membranes suggests that cellulose cushions fulfill the conditions of complete wetting of cell membranes. The simplicity of the method and the precise control of membrane orientation seem to result from the optimized cell-surface contact. However, it should be noted that FRAP experiments show no sign of the lateral diffusion of labeled band 3. This can be attributed to the presence of spectrin cytoskeletons,[28] to which band 3 is anchored via ankryn. The tracking of the lateral diffusion of single, individual proteins would be a possible solution to obtain the statistics of the diffusion coefficients of anchored/free proteins.[29] Cellulose cushions have also been used for spreading other native membrane extracts such as microsomes[30] as well as homogenized plasma membrane extracts,[31] which indicates a large potential of such an approach toward the creation of two-dimensional cell membranes on polymer supports. In addition to immune-

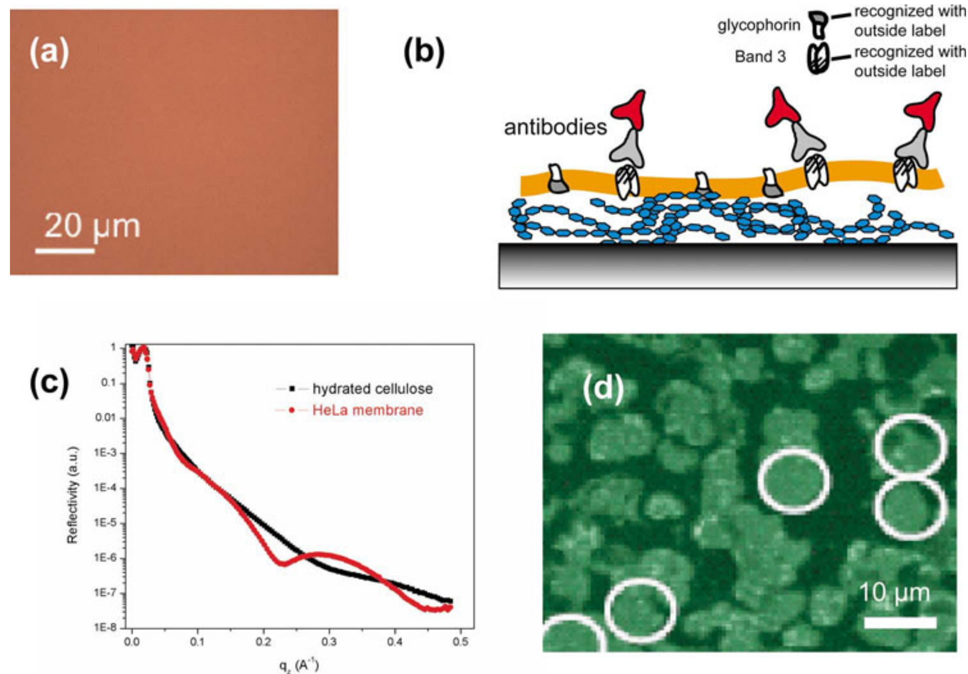


FIG. 3. (a) Immune-fluorescence image of a human erythrocyte membrane on a cellulose support (dry film thickness: 5 nm) and (b) a schematic illustration of the labeling with the first and second antibodies. (c) Specular x-ray reflectivity curves of a cellulose support (dry thickness: 5 nm) before (black) and after (red) the deposition of human carcinoma (HeLa) cell membrane extracts (Ref. 31). Clear minima observed after the membrane deposition imply the presence of a "layer" with a clear contrast in the electron density. (d) Immune-fluorescence labeling of the erythrocyte membrane on a poly(lysine) support (dry film thickness: 2 nm). Here, the cytoplasmic domain of band 3 is also labeled with the antibodies.

fluorescence labeling, detailed out-of-plane structures of native supported membranes are currently investigated with specular x-ray [Fig. 3(c)] [to avoid the absorption of x-ray in water, we carry out x-ray reflectivity at a high energy (22 keV) at the ID 10B beam line, European Synchrotron Radiation Facility (Grenoble, France)] and neutron reflectivity.

In contrast to the excellent compatibility of cellulose cushions to cell membranes, the incubation of ghost cells on poly(lysine) layers results in inhomogeneous patches of pinned membranes, which can be explained as the dewetting of negatively charged membranes on too highly attractive surfaces [Fig. 3(d)].[27] This indicates that polymer supports with stimuliresponsible physical properties can be used to fine adjust the "wetting" interactions between cell membranes and planar supports.[32,33]

V. CREATION OF MICRO-PATTERNS OF CELL MEMBRANES BY WETTING CONTRASTS

In case of solid-supported membranes, micrometer-sized patterns can be generated by deposition of diffusion barriers,[34,35] microstructuring of solid substrates,[36] photolithography,[37,38] direct microcontact printing of the membranes.[39] Such membrane micropatterns allow for cell growth in confined geometry,[40] while domain arrays can be used for activation of cells[41] and screening of antibodies or drugs targeting membrane proteins.[42]

The fact that cells do not adhere or spread on glass or quartz surfaces while cell membranes completely wet cellulose cushions suggests a unique possibility to selectively deposit cell membranes onto micropatterned polymer cushions. Micropatterns of cellulose cushions can be fabricated by deep UV photolithography. As we demonstrated in our previous account, human erythrocyte membranes can be spread on the area covered with cellulose, but the naked silica remains intact [Fig. 4(a)]. This is in clear contrast to the case of artificial lipid membranes, where the membranes often do not discriminate between different surfaces and can be deposited on patterns with even higher step heights.[41,43] Furthermore, the fact that the incubation of bovine serum albu-

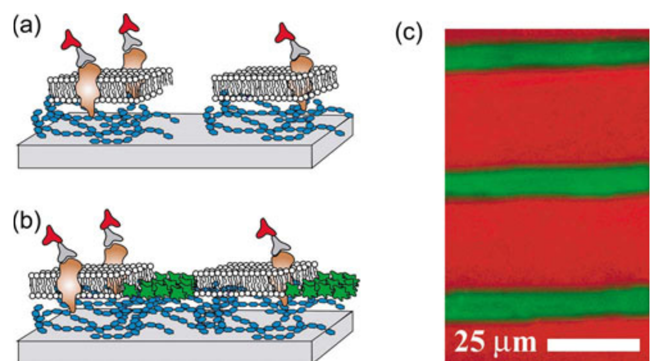


FIG. 4. (a) When cell membranes are incubated with micropatterned cellulose, cell membranes are selectively spread on the area coated with cellulose owing to the different wetting conditions. [(b) and (c)] Similar contrasts in wetting interactions can be introduced by microcontact printing of bovine serum albumin (labeled with green) on a cellulose cushion. Here, the membrane (labeled with red) also only spreads on the area exposing cellulose.

min (BSA) prevents the spreading of cell membranes on cellulose suggests that micropatterns of BSA on cellulose might also be used for position selective deposition of cell membranes. Actually, cell membranes do not spread over the area coated with BSA, but on the area coated with cellulose [Figs. 4(b) and 4(c)]. It should be noted that the thicknesses of cellulose films (5 nm) and that of BSA layers (2–3 nm) are by several orders of magnitudes smaller than the lateral dimension of the patterns (2–100 μm) and comparable to the thickness of cell membranes. This indicates that the micropatterns are not formed by step heterogeneity of the cellulose films or BSA layers, but rather the columnar heterogeneity corresponding to the contrast in wetting interactions.

As described above, ultrathin (thickness of 5–100 nm) polymer supports can be utilized not only for functional, stress-free deposition of artificial membranes with proteins but also for position selective deposition of native cell membranes and microsomes without loosing their natural compositions and orientations.[44] Scientific applications include the study on molecular level structures and physical principles of cell-cell and cell-tissue contacts, while the position and orientation selective deposition of native membrane micropatterns would be straightforward toward the creation of hybrid devices based on biomembranes and array-integrated semiconductor devices.[13,14] Thus, the polymer-supported membrane concept described in this review opens a large interdisciplinary breakthrough in membrane physics and biomaterials science.

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