Surface supported bilayer platform for studies of lateral association of proteins in membranes (Mini Review)

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Here, the authors review how surface supported bilayers can be engineered and how Förster resonance energy transfer (FRET) can be used to quantify interactions between transmembrane peptides in these bilayers. The requirements for the surface supported platform are (1) lateral mobility of the peptides, (2) transmembrane orientation of the peptides, and (3) capabilities for FRET measurements. To satisfy these requirements, a new assembly method, termed "directed assembly" was developed. This assembly method could have broad utility in basic studies of proteins in membranes and in biotechnological applications. © *2008 American Vacuum Society.*
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The past decade has brought significant advances in surface supported bilayer methodologies.[1–8] Here, we review how surface supported bilayers can be engineered and how Förster resonance energy transfer (FRET) can be used to quantify interactions between transmembrane (TM) peptides in these bilayers. The investigated TM peptides correspond to the TM domain of human fibroblast growth factor receptor 3, FGFR3 (TM_{FGFR3}), (wild-type sequence DEAGSVYAGILSYGVGFFLFILVVAAVTLCRLR). The whole-length FGFR3 receptor transduces biochemical signals across the plasma membrane via lateral dimerization, and its TM domain has a natural propensity to form sequence-specific dimers in the bilayer, such that the monomers and dimers are in equilibrium.[9,10] Furthermore, mutations in FGFR3 TM domain, such as the A391E mutation (in TM_{FGFR3}^{E}), induce pathologies by stabilizing the dimeric state.[11,12] Thus, measurements of free energies of dimerization of wild-type and mutant FGFR3 TM domains can shed light on the molecular mechanism behind the pathologies. This mini review describes a surface supported bilayer platform that has been used to study the thermodynamics of lateral interactions of TM peptides such as the TM domain of FGFR3.

First requirement

The first requirement for such a platform is the lateral mobility of the peptides. We have developed an assembly method termed "directed assembly" that yields bilayers with laterally mobile TM peptides. The novelty in this assembly approach is the incorporation of the peptides into the Langmuir-Blodgett (LB) monolayer at the first step of assembly. This approach allows precise control over the peptide concentration in the bilayer, the overall architecture of the structure, and the topology of the helices in the bilayer. To produce the bilayers, a lipid monolayer containing the peptides was deposited on a glass coverslip by the LB method. This step is followed by fusion of vesicles [no polyethylene glycol (PEG), extruded through 100 nm pore filters] as described.[13] Briefly, two clean wet coverslips were first stacked together and were vertically immersed into the clean subphase of a LB trough (model 611, Nima Technologies, Coventry, England). Next, a solution of lipids and fluorescently labeled peptides was spread dropwise at the air-water interface of the open trough (600 cm^2) [Fig. 1(A)]. After spreading, the solvents were allowed to evaporate for 30 min, and the monolayer was compressed to 32 mN/m. The monolayer was transferred on the outer surfaces of the coverslips during their withdrawal from the subphase at a rate of 15 mm/min at a surface pressure of 32 mN/m to form a supported LB monolayer [Fig. 1(B)]. The second lipid monolayer was deposited via vesicle fusion (VF), Figs. 1(C) and 1(D), as described.[13] Large unilamellar vesicles of ~100 nm diameter were used for the vesicle fusion step. Alternatively, Langmuir-Schaefer (LS) deposition was used to form the second leaflet of the bilayer. Bilayers, formed either by LB/VF or by LB/LS using this protocol, were stable for at least one day, once properly sealed to prevent dehydration.

The lateral mobility of peptides and lipids was measured using fluorescent recovery after photobleaching (FRAP) of NBD-labeled lipids and rhodamine labeled peptides. The diffusion coefficient was calculated using the boundary profile evolution (BPE) method.[13] This method allows quantitative diffusion coefficient measurements with a standard fluorescence microscope equipped with a mercury lamp. The bleached spot was set to ~100 μm and a sequence of images (every 5 min for peptides, every 20 s for lipids) was taken after bleaching. The time evolution of the intensity profile of the boundary region between bleached and unbleached areas, $F(x,t)$, is given by a Gaussian error function,[14]

$$2\frac{F(x,t)-F_{\text{bleached}}}{F_{\text{unbleached}}-F_{\text{bleached}}}=\text{erf}\left(\frac{x-x_b}{2w}\right)+1, \quad (1)$$

where F_{bleached} and $F_{\text{unbleached}}$ are the fluorescence intensities inside and outside of the bleached spot, x_b is the position of the boundary between the bleached and unbleached areas, and $(x-x_b)$ is the distance to this boundary. The diffusion depth w is defined as

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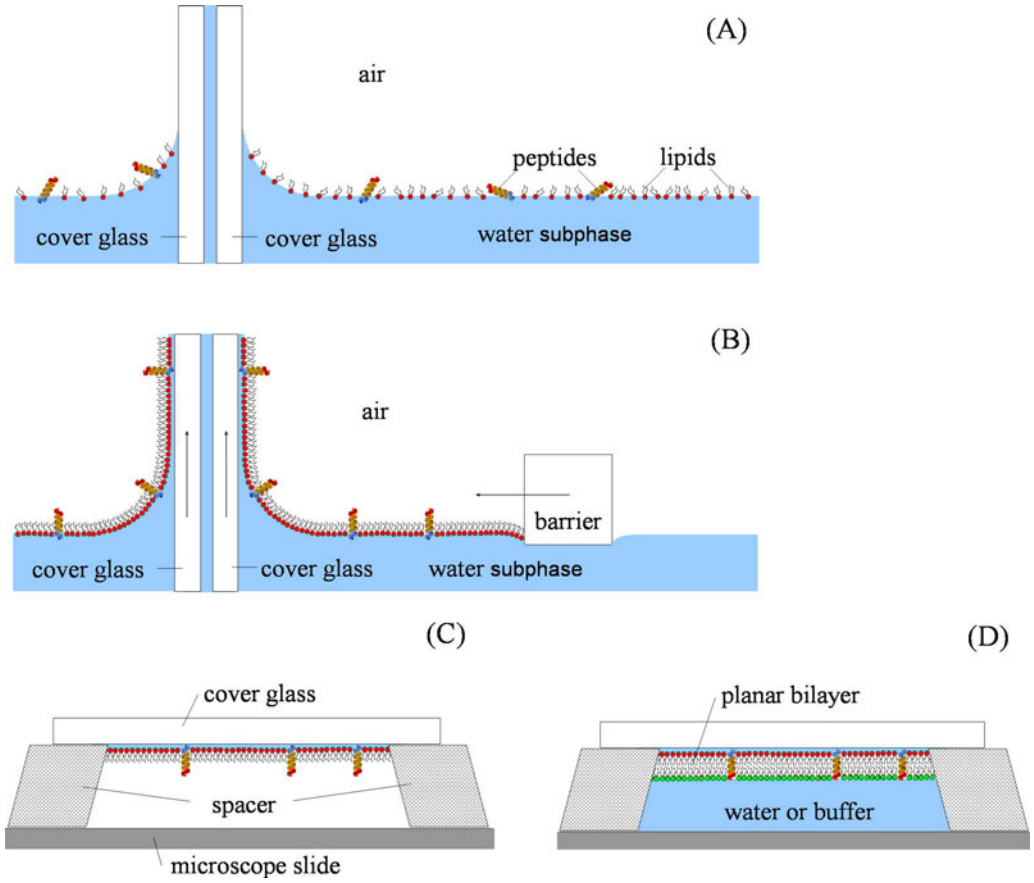

FIG. 1. Directed assembly of a surface supported bilayer with TM helices (not drawn to scale). (A) A mixture of lipids and peptides in chloroform and 2,2,2-trifluoroethanol is spread on the water/air interface. (B) After solvent evaporation, the monolayer is compressed and deposited on a glass coverslip using the Langmuir-Blodget method. (C) The coverslip is placed on top of a clean glass slide, with the monolayer facing the slide. (D) The monolayer is incubated with a solution of vesicles, the excess of vesicles is rinsed out, and the chamber is sealed.

$$w = \sqrt{Dt}, \tag{2}$$

where D is the diffusion coefficient and t is the lapse time after bleaching. Since $w^2 = Dt$, the slope of w^2 vs t is equal to the diffusion coefficients of peptides and lipids (see Fig. 2). The diffusion coefficients for lipids and peptides in bilayers composed of POPC, 0.5 mol % NBD-PE and 0.05 mol % peptides (1:2000 peptide-to-lipid ratio), were calculated as 2.3–2.7 and 0.006–0.007 $\mu m^2/s$, respectively.

The mobile fraction was measured by bleaching a 30 μm diameter spot and monitoring recovery over time.[13] The immobile fraction for both peptide and lipids, if existent, was less than 2% and could not be resolved. In all experiment, we observed a single population of slowly moving peptides. This allowed us to carry out the dimerization energetic measurements described below.

The BPE method offers advantages for both slow and fast moving molecules over the "traditional" half time of recovery trajectory. The advantage is obvious for the slow moving peptides, since the half time of recovery is hours (see Fig. 2 in Ref. 13), and therefore, experiments that measure recovery half times take many hours to complete (as compared to 10–20 min for the BPE method). For the fast moving lipids, the BPE method presents an alternative for quantitative diffusion measurements when state-of-the-art laser-based FRAP or single molecule setups are not available. The BPE method works even if the bleaching time is comparable to the recovery time in the FRAP experiments, allowing us to conduct experiments on a standard fluorescence microscope without a laser. Note that the traditional half-time recovery method gives the correct diffusion coefficient only if the bleaching time is much shorter than the recovery time.[15–17]

The lipid diffusion coefficients calculated using the BPE method are very similar to previously reported values obtained using other methods, such as flash bleaching and single molecule measurements.[18] The diffusion coefficients measured for the peptides, however, were very low. We therefore explored whether the incorporation of a PEG cushion between the bilayer and the substrate increases protein mobility.

The PEG cushions were incorporated by adding PEG lipids to the protein/lipid monolayer at the air/water interface, at the first step of bilayer assembly. All LB monolayers (monolayers facing the support) contained POPC as the host component, 1 mol % NBD-PE, 0.1 mol % peptide, and PEG lipids. The concentration of the PEG lipids in the LB monolayer was chosen as multiples of the crossover concentration,

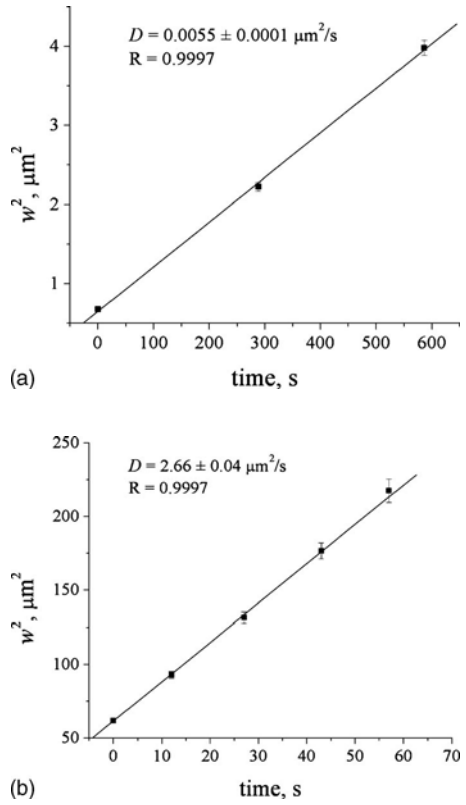

FIG. 2. Measurements of peptide diffusion coefficients using the boundary profile evolution (BPE) method described in (Ref. 13) for peptides (A) and lipids (B). The diffusion coefficient D is determined from the plot of w^2 vs t. Since $w^2=Dt$, the slope of the line is equal to D. Data are from (Ref. 13).

ξ, which is a function of the molecular weight of the PEG chain. The concentrations used were 0.25ξ, 0.5ξ, ξ, 2ξ, and 4ξ. The crossover concentration ξ marks the transition from the "mushroom" to the "brush" regime[19] and describes the case when the PEG random coils are barely touching each other. The size of the random coil is given by the Flory radius, and at the crossover concentration, ξ, the average spacing between the PEG lipids in the LB monolayer is equal to the Flory radius. The mole fraction of PEG lipids at the crossover concentration, ξ, was 14%, 5.9%, 3.7%, and 2% for PEGs of molecular weight of 1000, 2000, 3000, and 5000, respectively.[20]

Diffusion coefficients were measured as a function of PEG-lipid concentration and PEG chain length and means of attachment to the surface (see Fig. 3). At least four different bilayers, prepared under identical conditions, were characterized and the mean diffusion coefficients are shown in Fig. 3. There were no systematic changes in diffusion coefficients upon variations in PEG length and concentration, demonstrating that the PEG cushion is neither a necessary nor a required component in the assembly of surface supported bilayers with mobile transmembrane peptides. Contrary to our expectations, the incorporation of a PEG cushion did not increase the low peptide mobility in the surface supported bilayers produced via directed assembly. For the highest PEG molecular weight and the highest PEG-lipid concentration, the distance between the substrate and the bilayer should exceed 10 nm. Therefore, there should be no physical contact or even long-range interactions between the substrate and the proteins in the bilayer. Thus, it appears that the low protein mobility is not due to protein-substrate interactions, but is intrinsic to the bilayer itself.

Second requirement

The second requirement for the platform is that the proteins are transmembrane (TM). The TM orientation of the helices is confirmed using oriented circular dichroism (OCD). For OCD measurements of fluid bilayers, 26 monolayer-coated slides were stacked between two clean quartz slides, and placed in the spectropolarimeter cuvette

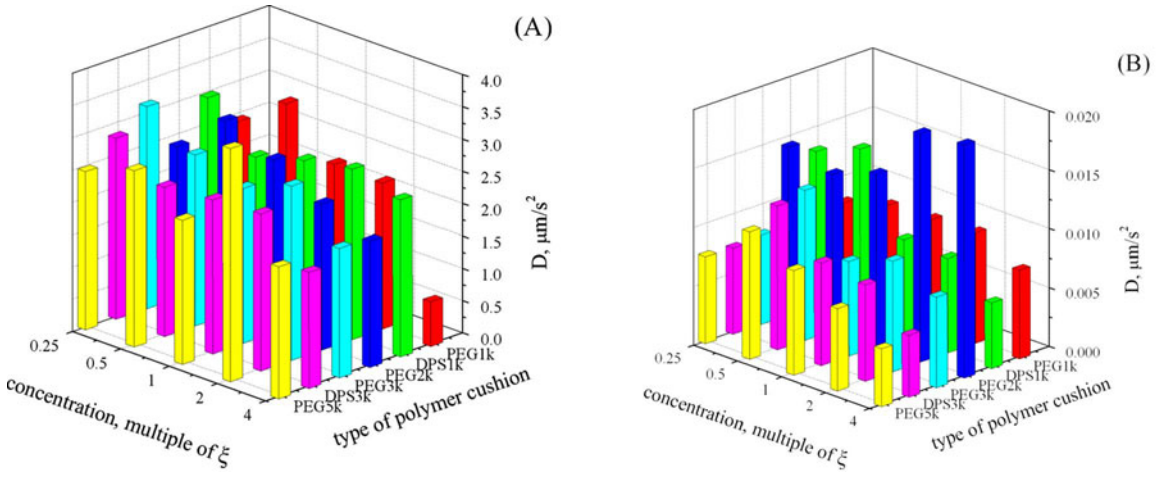

FIG. 3. Diffusion coefficients of NBD-PE (A) and Cy3-TM_{FGFR3} (B) in supported bilayers prepared via the directed assembly method (LB/LS deposition) at different PEG lengths and different PEG-lipid concentrations. The PEG chain length was varied from 1000 Da (in PEG1k) to 5000 Da (in PEG5k). The PEG chains were either chemically tethered to the surface via a silane moiety (in 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[propylmethyldimethoxysilyl (polyethylene glycol)-1000] (DPS1k) and in DPS3k), or physically adsorbed. The crossover concentration ξ corresponds to the transition between polymer mushrooms (at low PEG-lipid concentrations) to a polymer brush (at high PEG-lipid concentrations). Data are from (Ref. 34).

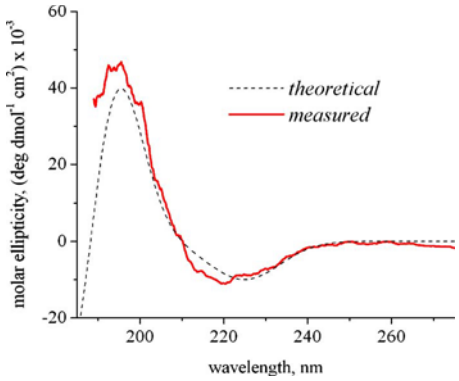

FIG. 4. Oriented circular dichroism (OCD) spectrum of TM_{FGFR3} in surface supported fluid bilayers. Comparison of the measured spectrum with the theoretical one indicates that the orientation of TM_{FGFR3} is transmembrane. Data are from Ref. 13.

holder such that they were normal to the beam, with the beam passing through the center of each slide. POPC vesicles were added in between the slides and incubated for 5 min. The excess vesicles were removed by rinsing with deionized water. Thus, a total of 52 fluid supported bilayers were formed, each oriented normal to the beam. Ten different CD spectra were recorded and averaged. Background CD spectra were collected from bilayers composed of lipids only, and subtracted from the peptide/lipid spectra.

The OCD signal is dramatically different for helices that are normal and parallel to the beam, such that TM orientation is easy to confirm once a high quality OCD spectrum is available. Figure 4 shows the predicted OCD spectrum for helices that are normal to the bilayer plane.[21] It exhibits a single minimum around 230 nm and a maximum around 200 nm.[21] The OCD spectrum of a helix that is parallel to the membrane plane, however, exhibits two minima at 205 and 225 nm and a maximum around 192 nm. The experimental OCD spectrum of TM_{FGFR3} in supported bilayers, collected from 52 bilayers stacked between 28 slides (solid line in Fig. 4) shows a single minimum, indicating that the peptides are normal to the bilayer plane. The amplitude of the measured TM_{FGFR3} spectrum is also very similar to the theoretical one,[21] further confirming the TM orientation of the peptides.

Third requirement

The third requirement for the surface supported platform is that FRET can be measured in a single bilayer. This was achieved by recording emission and excitation spectra in a Fluorolog-3 fluorometer (Jobin Yvon, Edison, NJ). The quartz slide, with the coverslip supporting the bilayer, was inserted into the homebuilt adapter as described.[22] The interface reflection of the excitation beam was diverted from the photodetector optical line, allowing measurements of undistorted emission spectra. This experimental setup has allowed us to compare spectra of fluorescein and rhodamine labeled peptides in bilayers produced via the directed assembly method to spectra of fluorescein and rhodamine labeled peptides in suspended liposomes. The spectra in the surface supported bilayers (i.e., close to the surface) are very similar to spectra in liposomes,[22] demonstrating the feasibility of FRET measurements in single supported bilayers.

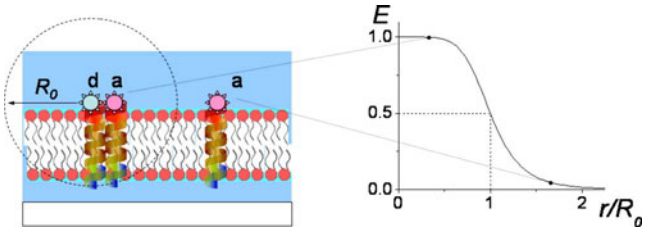

FIG. 5. Schematic drawing of donor- and acceptor-labeled TM helices in supported bilayers (drawn approximately to scale) and the corresponding FRET efficiencies. The distance between the FRET pair in the dimer is well within the Förster radius, $R_0 \sim 55$ Å, resulting in almost 100% FRET efficiency.

FRET involves the nonradiative transfer of energy from the excited state of a donor to an appropriate acceptor.[23–27] In a TM dimer with a donor and an acceptor, the two dyes are 10–20 Å apart,[9] and energy is effectively transferred to the acceptor when the donor is excited[10] (see Fig. 5). The measured FRET efficiency can then be used to characterize interaction energetics in bilayers.[10]

To determine if only monomers and dimers exist in the bilayer, we measured FRET efficiencies as a function of acceptor fraction, as previously described.[11,28,29] The total peptide concentration was fixed, while the donor-to-acceptor ratio was varied. The measured FRET efficiencies, calculated from the decrease in donor fluorescence,[10] are shown in Fig. 6. The presence of larger aggregates leads to a nonlinear dependence of the FRET efficiency on the acceptor mole fraction (discussed in Refs. 10 and 30). The observed linear dependence in Fig. 6 indicates that the peptides in the surface supported bilayer exist in a monomer-dimer equilibrium and do not form larger aggregates.

Having proven that the peptides form dimers, we next recorded FRET spectra of fluorescein and rhodamine labeled

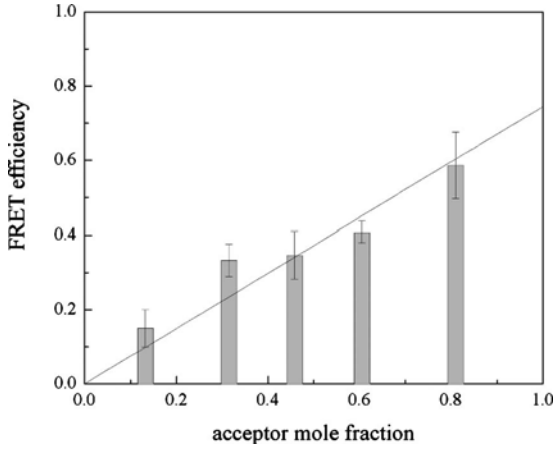

FIG. 6. Measured FRET efficiency for TM_{FGFR3}^{E} as a function of acceptor fraction. The dependence of the FRET efficiency on the acceptor mole fraction is linear, indicating that only monomers and dimers, but no large peptide aggregates, exist in the supported bilayer (Ref. 22).

peptides at 1:1 donor-to-acceptor ratio, while varying the total peptide concentration.[22] The measured FRET efficiencies, calculated from the decrease in donor fluorescence, were used to determine the monomer and dimer fractions, $[D]$ and $[M]$, as described previously.[10] These calculations required that the FRET contribution from random colocalization of donors and acceptors is subtracted from the measured FRET efficiency, as described in Refs. 10 and 11. Statistical arguments for the occurrence of donor-donor, acceptor-donor, and acceptor-acceptor dimers, were also taken into account for the calculation.[9,10] The association constant K was calculated as $K=[D]/[M]^2$, and the free energy of dimerization was determined as $\Delta G=-RT\ln \mathrm{K}$.[10] The dimerization free energy, calculated from the FRET efficiencies is -3.6 ± 0.6 kcal/mol, identical to the previously published value, -4.0 ± 0.2 kcal/mol, for the same $\mathrm{TM}_{\mathrm{FGFR3}}^{\mathrm{E}}$ peptide measured in liposomes.[11]

There are advantages to performing the measurements in supported bilayers, as compared to free liposomes in suspension: (1) the amount of peptide required for an experiment in surface supported bilayers, produced via directed assembly, is 1/100 of the peptide amount typically used in vesicle solutions, thus substantially reducing the cost of research due to the very high cost of the chemically synthesized and labeled TM peptides. (2) The directed assembly method allows the assembly of asymmetrical surface supported bilayers, composed of two leaflets with different lipid compositions. (3) Supported bilayer platforms, unlike vesicle solutions, could be adapted to parallel high-throughput measurements of lateral protein interactions in bilayers, paving the way for the development of novel sensing devices that utilize membrane proteins.[1,2]

ACKNOWLEDGMENTS

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