Published in final edited form as:

Langmuir. 2013 January 8; 29(1): 299–307. doi:10.1021/la303628n.

Tether-Supported Biomembranes with Alpha-Helical Peptide-Based Anchoring Constructs

Lina Zhong[✦], Raymond Tu[✦], and M. Lane Gilchrist^{✦,§}

[✦]Department of Chemical Engineering, The Grove School of Engineering, The City College of New York, 140th Street @ Convent Ave, New York NY 10031

[§]Department of Biomedical Engineering, The Grove School of Engineering, The City College of New York, 140th Street @ Convent Ave, New York NY 10031

Abstract

The strict requirement of constructing a native lipid environment to preserve the structure and functionality of membrane proteins is the starting constraint when building biomaterials and sensor systems from these biomolecules. In order to enhance the viability of supported biomembranes systems and build new ligand display interfaces, we apply rationally-designed peptides partitioned into the lipid bilayer interface. Peptides designed to form membrane-spanning alpha-helical anchoring domains are synthesized using solid-phase peptide synthesis. K₃A₄L₂A₇L₂A₃K₂-FITC is synthesized at 100 mg scale for use as a biomembrane anchoring molecules, where orthogonal side-chain modifications allows us to introduce probes enabling peptide localization within supported bilayers. The peptides are found to form alpha-helical domains within liposomes as assessed with circular dichroism spectroscopy. These peptides are designed to incorporate into lipid bilayers supported by microspheres and serve as biomembrane anchoring moieties to amino-terminated surfaces. Here, the silica bead surface (4.7 μm diameter) is activated with homobifunctional NHS-PEG₃₀₀₀-NHS as “polymer cushion” spacers. This tethering to a subset of the K₃A₄L₂A₇L₂A₃K₂-FITC molecules present in the bilayer is achieved by fusion of liposomes followed by coupling of the peptide amino groups to the NHS presented from the silica microsphere surfaces. The biomembrane distributions of tethered and untethered K₃A₄L₂A₇L₂A₃K₂-FITC is probed with confocal microscopy and found to give 3D reconstructions consistent with largely homogeneous supported biomembranes. The fluidity of the untethered fraction of the peptides within supported membranes is quantified using the fluorescence recovery after photobleaching (FRAP) technique. The presence of the PEG₃₀₀₀ polymer cushion facilitated a 28.9% increase in peptide diffusivity over untethered bilayers at the lowest peptide to lipid ratio we examined. We show that rationally designed peptide based anchors can be used to tether lipid bilayers, creating a polymer cushioned lipid microenvironment on surfaces with high lateral mobility and facilitating the development of a new platform for ligand displays.

Keywords

Supported Membranes; helical peptides

*To whom correspondence should be addressed: Lane Gilchrist: gilchrist@ccny.cuny.edu Phone: 212-650-6664 or Raymond Tu: tu@ccny.cuny.edu. Phone: 212-650-7031.

Author Contributions. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information.

Extended description of the methods of analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Provided that the lipid composition is similar to that found in the native microenvironment, the activity of membrane proteins can be rejuvenated after they are reconstituted into lipid bilayers. Moreover, lipid bilayers are often tethered to solid supports to create model systems for biological membranes.^{1, 2} One widely used method is to prepare supported membranes and incorporate ‘anchor’ molecules with functional proteins coupled to those anchors.¹ Supported membranes are readily obtained by fusing liposomes onto hydrophilic solid surfaces with a water-laden substrate to bilayer spacing of 5–20 Å.^{1, 3–6} This distance is usually not sufficient to provide an aqueous layer large enough to accommodate the hydrophilic domains of moderate-sized membrane proteins or their lateral transport, resulting in surface “pinning” that leads to loss of mobility and often function. This drawback can be overcome by separating the membrane from the solid substrate using a tethering spacer arm composed of soft polymeric materials,^{7–11} in which “polymer cushioning” ideally acts as a lubricating layer between the membrane and the substrate. The spacers can be based on a wide range of compounds, including oligo(ethylene oxide) and poly(ethylene oxide)^{10, 12, 13} and oligopeptides with thiol groups.¹⁴

Supported membranes on microparticles, termed lipobeads, are mechanically stable compared with lipid vesicles and easily adapted to various functions.^{3, 15–17} It is crucial to preserve the lateral fluidity of the lipids and membrane proteins within the supported membranes, as well as impart thermodynamic and mechanical stability to these colloidal assemblies. Instead of using proteins as ‘anchor’ molecules, alpha-helix peptide anchors have some advantages, including the ability to manipulate the biomembrane with external perturbations such as photoactivation,¹⁸ to stabilize the membrane as a function of peptide sequence and size,¹⁹ and to conjugate the peptides with various reporter groups. Using fluorophore labeling enabled by solid-phase synthesis, we can study alpha-helical peptide lateral mobility and anchor localization in the supported membrane structures with fluorescence microscopy.

Anchored lipid assemblies that contain membrane proteins were first introduced by Rothe and Aurich using polyacrylic beads.²⁰ In subsequent studies, Schmidt et al. achieved successful fusion of liposomes with reconstituted receptor onto a gold-supported thiopeptide lipid monolayer and used surface plasmon resonance spectroscopy (SPR) to monitor in the fusion process in real time at the surface. In their work, the use of fluorophore-labeled antibodies in combination with SPR was shown to be a useful tool for the investigation of binding processes on surfaces.²¹ Giess et al. introduced solid-supported tethered bilayer lipid membrane (tBLM) for the functional incorporation of membrane proteins, based on Ni²⁺ chelation where His-tagged cytochrome c oxidase (CcO) was used as a model protein, opening new prospects for the investigation of functional membrane proteins by various surface-sensitive techniques under a defined electric field.²² The creation of a membrane protein-containing BLM is completed by replacing detergent molecules of the bound protein by lipids in a reconstitution step, thus forming lipid bilayers around the tethered membrane protein molecules. In the work of Bunjes et al., reactions were monitored at solid/solution interfaces by fusion of vesicles prepared from a fluid lipid mixture with and without reconstituted proteins. These studies gave rise to an ideal tethered bilayer model with the successful incorporation of active ATPase into these membrane matrices.¹⁴ In other studies, inherently high levels of lateral mobility are designed into supported lipid bilayer systems with novel mobile liquid mercury substrates, successfully used to incorporate the HERG potassium channel and other membrane proteins.^{23, 24} In our previous studies, bacteriorhodopsin was used as an anchoring construct to build fluid, tether-supported bilayers on microspheres.²⁵ This aim of this investigation is to harness the high level of

molecular structural control of peptides to extend the concept of using alpha-helical domains as versatile anchoring elements in tether supported bilayers.

Our aim is to use alpha-helical peptide complexes as anchoring constructs to enhance biomembrane stability to mechanical challenges through tethering and to form elements for building ligand displays on biomembrane surfaces. In this work, we have synthesized and characterized a fluorescent alpha-helical peptide derivative, $K_3A_4L_2A_7L_2A_3K_2$ -FITC, designed for use as an anchoring construct for tethered lipid bilayers. To probe the peptide helical structures formed within liposomes, circular dichroism spectroscopy was used. By using such fluorophore-labeled peptides as anchoring constructs, we are able to employ confocal microscopy-based methods to monitor their localization and lateral mobility within supported biomembranes. This study outlines a route for use of these molecules as building blocks for supported biomembrane systems that allow for the *in situ* localization of mobile or anchoring peptides and exploit the versatility of solid phase peptide synthesis in advancing the design of supported biomembrane ligand displays.

MATERIALS AND METHODS

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Fluorescein isothiocyanate (FITC) and 6-Aminofluorescein were purchased from Thermo Scientific Corp (Chicago, IL) (5-(and 6-carboxytetramethylrhodamine succinimidyl ester), (5, (and 6)-carboxylfluorescein, succinimidyl ester), N,N-dimethyl formamide (DMF), Dichloromethane (DCM), trifluoroacetic acid (TFA, 95%), Methyl tert-butyl ether (MtBE), Methanol, and Ethanol were obtained from Fisher Scientific (Chicago, IL) and all solvents used were reagent grade. DiNHS-PEG₃₀₀₀ was obtained from Rapp Polymere (Dusseldorf, Germany). The polydispersity index (PDI) for this polymer was < 1.08. Fmoc-Lys(Dde)-OH was obtained from Anaspec, Inc. (Boston, MA). Silica beads terminated with amino groups of 4.7 μ m nominal size were obtained from Bangs Laboratories (West Bend, IN). FluoSpheres™ Fluorescent Carboxylate-Modified Microspheres, 0.04 μ m were obtained from Life Technologies, Inc. (Carlsbad CA).

Solid Phase Peptide Synthesis

Solid Phase Peptide Synthesis using Fmoc chemistry was used in peptide synthesis. An orthogonal labeling strategy using the protected lysine derivative, Fmoc-Lys(Dde)-OH (Dde: (4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidine)ethyl), was used to add a single amino conjugation than was labeled with FITC. We synthesized the peptide $K_3A_4L_2A_7L_2A_3K_3$, using a PS3 peptide synthesizer, then added the lysine derivative, fmoc-Lys(Dde)-OH onto the N-terminus manually. The N-terminal Dde group is more susceptible to removal on either prolonged or repeated treatment with 2% hydrazine in DMF, subsequently the reporter group FITC was conjugated at the terminal lysine ϵ -amino group.

Fluorophore labeling

For this study, we chose fluorescein isothiocyanate as the fluorescent tag because signal calibration microspheres loaded with this dye are commercially available. After the N-terminal Dde group is removed, we labeled the orthogonal site by incubating the resin-bound peptide with FITC at room temperature overnight, keep reaction in dark. Later the tBoc group is removed from peptide amino groups and the peptide is cleaved from the resin by a mixture of 95% trifluoroacetic acid (TFA). The peptide precipitates in methyl tert-butyl ether (MtBE) and we purified using multiple precipitation/centrifugation steps.

HPLC-MS characterization and HPLC purification

Resin-cleaved sample aliquots were taken after the N-terminal Dde FITC conjugation. ESI-MS experiments were performed on an 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA) with an ESI (electrospray ionization) ion source interfaced with a Shimadzu Prominence HPLC (Shimadzu USA, Canby, OR). The HPLC Column used was a Zorbax Eclipse XDB-C18 (4.6mm × 150mm × 5μm) and the mobile phase consisted of A: Water-0.1% formic acid, B: Methanol -0.1% formic acid. The peptide purity was estimated by observation of peak areas using Analyst Software (Applied Biosystems, Foster City, CA) and Igor Pro 6.0 (Wavemetrics Inc., Portland, OR)

Circular Dichroism

The circular dichroism spectra are collected using a Olis DSM 20 spectrophotometer (Olis, Bogart, GA). CD spectra were obtained in PBS pH 7.4 buffer in peptide liposomes at 1 mM concentration. Estimation of protein secondary structure was carried out using basis spectra from model polypeptides to form linear combination fits with the lowest root-mean-squared-deviation (RMSD).

Peptide Proteolipobead Construction

A flowchart of peptide proteolipobead formation is shown in Figure 1. The peptide supported lipid bilayers are formed by liposome fusion at microsphere interfaces, starting with a dried peptide/lipid film containing specified lipid to peptide molar ratios. We hydrated the peptide/DOPC lipid films in PBS buffer, vortex mixed the dispersion, and then probe sonicated the dispersion in an ice-cold bath to yield peptide proteoliposomes. We studied two distinct cases; **(1) polymer cushion tethered peptide proteolipobeads** in which a fraction of the peptides were tethered and the majority were laterally mobile and **(2) untethered supported peptide proteolipobeads** which were suspended above the silica surface by a thin water layer. For the spacer-tethered sample, we functionalized amino-terminated 4.7 μm silica beads with NHS-PEG₃₀₀₀-NHS for 90 minutes at room temperature to yield PEG₃₀₀₀-NHS activated substrate beads. This incubation time was determined via flow cytometry of PEG₃₀₀₀-NHS activated microspheres labeled with 6-aminofluorescein, described in Figure S-1 of the supplemental data. The flow cytometry was conducted using a BD Biosciences FACSCalibur flow cytometer, with 488 nm excitation and mean channel fluorescence in the FL1 channel (500–550 nm) as the readout. The final step was to incubate the functionalized beads and control non-functionalized beads with peptide liposomes for 2 hour at room temperature for crosslinking from surface PEG-NHS groups to peptide amino groups. The ratio of peptide liposome to microsphere surface area in the fusion steps was 5:1. Finally, after washing away the excess liposomes we obtained the pPLBs, lipid bilayers supported by microspheres, both tethered with PEG₃₀₀₀ polymer cushions and untethered.

Confocal Microscopy

Confocal microscopy was used to image the supported biomembrane structures on the bead surface and the mobility of the peptides within these structures was measured using confocal-FRAP. The samples were imaged using a Leica TCS SP2 AOBS confocal microscope system equipped with argon ion and HeNe lasers. A 63×/1.4 NA oil immersion objective was used for all the images. FITC was excited using the 488 nm line of the argon laser, and images were taken with the detection window set between 500 and 615 nm. The pinhole aperture was set at an Airy value of 1.0, which was equivalent to sampling a 500 nm vertical z-slice of the lipobead, as estimated by the axial resolution, $r_{z,\text{confocal}} \approx 1.4 \lambda_{\text{em}} n / \text{NA}^2$ (NA: numerical aperture, n : refractive index, λ_{em} : emission wavelength (525 nm)).²⁶ Sub-resolution polystyrene nanospheres of 40 nm diameter (FluoSpheres® Fluorescent

Carboxylate-Modified Microspheres) were used to observe intensity point-spread functions in the confocal microscope and monitor and maintain calibration of the 3D imaging.

The heterogeneity of fluorescent structures on the microspheres was examined by analyzing the equatorial sections of the microspheres as sets of 20 or more randomly selected assemblies. Global thresholding was used within the Leica Microsystems suite of image analysis tools to enhance and profile heterogeneous regions so their frequency of occurrence and nominal size could be measured manually and tabulated (version 2.5). In a smaller number of microspheres, lipid bilayer 3D reconstruction was conducted using Amira 3.1, using the Projection view display and Voltex rendering routines.

Fluorescence recovery after photobleaching (FRAP) studies were carried out using built-in protocol of Leica SP2 AOBs system. The image plane was set at the equator of the bead and a 512×32 pixel format was used (zoom value 16; scan speed 400 Hz, 488 nm AOTF 2%). This enabled the fast imaging (0.2 sec/scan point) of two equatorially opposite ends of the bead. After 5 pre-scans a region of $1\mu\text{m} \times 1\mu\text{m}$ on the bead was subjected to bleaching laser intensity for the duration of one scan point. This resulted in bleaching of the selected region on the proteolipobead sample. Recovery of fluorescence was monitored for >80 sec at normal laser intensity (AOTF 2%). Data was collected for the normalized fluorescence intensity of the bleached region throughout and analyzed using Mathematica to estimate the value of the mobile fraction, α , and the diffusion coefficient ($\mu\text{m}^2/\text{s}$), as shown in Figure S-3 in the supplemental data section.^{27, 28} The FRAP data was not collected in regions adjacent to defects or inhomogeneities in the supported biomembrane.

RESULTS AND DISCUSSION

The target peptide structure was selected based on extensive earlier studies of alpha-helix forming sequences by Bechinger and coworkers and other groups.^{29, 30} We chose to synthesize $\text{K}_n\text{A}_4\text{L}_2\text{A}_7\text{L}_2\text{A}_3\text{K}_n$ -based peptides, previously shown to form stable alpha-helical spanning in lipid bilayers and thus good candidates for biomembrane anchoring constructs. The first characterization method of our target α -helical peptide was undertaken using ESI-MS coupled to HPLC. Figure 2 displays the ESI-MS spectrogram of the FITC-labeled species, obtained after Fmoc-Lys(Dde)-OH addition, subsequent deprotection of the terminal e-amino group of lysine and FITC labeling at site. The main species (*) obtained exhibited m/z value of 392.2 m/z, with six positive charges and a calculated molecular weight of 2349.6 g/mol, consistent with the species $\text{K}_3\text{A}_4\text{L}_2\text{A}_7\text{L}_2\text{A}_3\text{K}_2$ -FITC. The conjugated species was isolated to greater than 85% purity. The unlabeled peptide peak at 257.4 m/z is denoted by (**). From this data, we conclude that the conjugation was successful and the peptide synthesis route gave the desired FITC-labeled species. (Figure 1)

To probe the peptide structure, we employed circular dichroism spectroscopy of the FITC-conjugated peptide in liposomes. Helical structures have been observed using NMR methods for peptides of similar structure,^{29, 31} with the peptides spanning lipid bilayers in a single pass helix described as a type I membrane protein domains, similar to many domains found in cell surface proteins such as glycoporphin and transmembrane domains of growth factor receptors.³² Figure 3 displays the CD spectrum (green curve) obtained from the $\text{K}_3\text{A}_4\text{L}_2\text{A}_7\text{L}_2\text{A}_3\text{K}_2$ -FITC species embedded in liposomes. In this case, minima are seen near 225 nm that are indicative of high α -helical content. These negative peaks found at 209 and 222 nm present are characteristic of helix formation.^{31–33} Secondary structure analysis of the FITC-labeled peptide yielded high α -helicity in liposomes. The red dotted line in Figure 3 shows our secondary structure fit corresponding to 80.7% alpha-helix and 19.3% random coil. This is in good agreement with what might be expected, as the five flanking residues are lysines (~21%) outside of the predicted alpha-helical hydrophobic core that matches the

size of the biomembrane hydrophobic region. The helicity of this species could be expected to be somewhat lower as the effect of FITC labeling likely perturbed the peptide structure, as the FITC group constituted a greater than 20% increase in molecular weight, adding a sizeable hydrophobic moiety to the N-terminal residue of the molecule. It is unclear if at our peptide to lipid ratio the peptide aggregates, perturbing the intramembrane structure relative to freely diffusing species and giving a minor fraction of peptides present in a random coil conformation.

Using liposome formulations similar to those used in the CD studies, peptide proteolipobeads are constructed via liposome fusion onto 4.7 micron amino-terminated silica microspheres. Two types of proteolipobead systems were examined; **(1) polymer cushion tethered peptide proteolipobeads** connected to the substrate via homobifunctional crosslinking from surface amines to peptide lysines via NHS-PEG₃₀₀₀-NHS and **(2) control untethered peptide proteolipobead** with a thin water layer separating the biomembranes from the substrate (see Figure 1). In the tethered case the fusion was initiated at 90 minutes past the beginning of the incubation of amino-terminated microspheres with NHS-PEG₃₀₀₀-NHS, determined to be the optimal time in flow cytometry based studies of PEG-NHS activation, as shown in the supplemental data Figure S-1. Figure 4 illustrates representative 3D reconstructions from CLSM of lipopeptide bead supported lipid bilayers consisting of DOPC and the K₃A₄L₂A₇L₂A₃K₂-FITC species. The data shown was collected at peptide to lipid mass ratios of 1:10 in the untethered (Figure 4A) and PEG₃₀₀₀ tethered case (Figure 4B). Both representative reconstructions show highly uniform supported bilayer structures with some surface inhomogeneities (white arrows). The estimated peptide area fraction of the supported biomembrane at the 1:10 mass ratio is approximately 8.5%, based on molecular structure arguments of the ratio of alpha-helix area to lipid area, reducing down to ~4.3% at the 1:20 mass ratio.

An in-depth analysis of surface coverage and biomembrane defect levels was conducted based on the 3D reconstructions of N=20 randomly selected microspheres of each type, examining the lipobead structures obtained using peptide to lipid mass ratios of 1:10, 1:15 and 1:20. This data is summarized in figures 4C-D. In both the tethered and untethered case, the coverage was greater than or equal to 88%. The coverage is slightly lower in the polymer-cushioned tethering case and is not strongly dependent on peptide to lipid mass ratio since the total liposome to microsphere surface area ratio is in greater than five-fold excess in the fusion step. Figure 4D is the summary of the average number of brighter fluorescent inhomogeneities found on the lipobead surface, determined using the method described in supplemental data figure S-2. These defects (figure 4 A-B, white arrows) are attributed to aggregated peptide or unfused peptide liposomes. The number of defects decreases when the peptide to lipid mass ratio is decreased and the “in membrane concentration” is two fold lower (1:10 to 1:20). This is an expected outcome of peptide aggregation. Given that the average size of the defects is ~0.8 microns, the fraction of the pPLB surface that is defect-free is ~90% (or greater than 94% in the 1:20 case). The even signal intensity levels arising from multiple pPLB assemblies is consistent with largely single continuous supported bilayer formation, further bolstered by the peptide diffusivity measurements outlined below.³

If the time delay after NHS-PEG₃₀₀₀-NHS functionalization of the amino-terminated microspheres was extended until the terminal NHS groups were deactivated by hydrolysis (>5 hours), liposome fusion was minimal and sparse (data not shown). This is consistent with our earlier work with microspheres of this type that were functionalized with PEG₃₄₀₀-biotin chains, in this case near complete passivation to liposome fusion was evidenced and negligible amounts of supported lipid bilayers were formed.²⁵ We note that there have been numerous 2D supported bilayer applications where the underlying polymer cushion is not

tethered. In these cases, liposomes are fused to a 2D PEGylated surface or PEGylated liposomes are fused to a 2D hydrophilic surface.³⁴ However in our systems, in 3D assembly conditions with silica microspheres of this size in well-mixed solutions, PEGylation of the silica microsphere surface inhibits fusion of liposomes relative to the near complete coverage that is evidenced with bare silica surfaces. To quantify the NHS hydrolytic degradation, the time dependent conjugation of 6-amino-fluorescein was examined, confirming the timescale of NHS hydrolysis (see supplemental data figure S-1). Taken together, this data is consistent with the formation of homobifunctional crosslinks to give microsphere-PEG₃₀₀₀-K₃A₄L₂A₇L₂A₃K₂-FITC tethering, where the peptide serves as an anchoring construct in polymer cushioned supported biomembranes. The approximate polymer cushion spacing between the silica surface and tethered biomembrane is 4.4 nm, as calculated from the Flory radius of PEG₃₀₀₀ ($R_F = aN^{3/5}$; $N_{Avg} = 68$ and $a = 0.35$ nm for -CH₂CH₂O-: “mushroom regime”).

To characterize the lateral mobility of the supported bilayers formed, we conducted confocal-based fluorescence recovery after photobleaching (FRAP) studies. We note that in these samples each contain a mixture of mobile FITC-labeled peptides of both inside-out and outside-in orientation. The diffusion coefficient can be determined as is outlined in the supplemental data section and Figure S-3.²⁷ We measured the mean value of the half time of recovery, calculating the value of the diffusion coefficient and recovery percentage denoted as the mobile fraction, α . Figure 5 displays tabulated results for FRAP measurements at the equatorial z section from a random selection of lipobeads ($N = 40$); the peptide to lipid mass ratios are indicated. The results from the tethered, polymer-cushioned case are given in the diagonal patterned bars (left; blue) and the untethered proteoliposomes are indicated by the dotted pattern bars (right; red). The mean diffusion coefficients are 0.0161 ± 0.0006 for the untethered sample versus $0.0192 \pm 0.0007 \mu\text{m}^2/\text{s}$ for the tethered, polymer-cushioned sample, a greater than 19.2% increase due to the effect of polymer cushioning (1:10 peptide to lipid mass ratio). This difference increased to 28.9% at the 1:20 peptide to lipid mass ratio. At the levels of peptide loading we examined, the diffusivity was significantly higher in the polymer-cushioned case, with p-values of $p < 0.001$ in each case. This is not unexpected, as there are a wide range of precedents for the positive influence of polymer cushioning on membrane protein mobility and function in supported membranes,^{7, 10, 34} To put the significance of this 28.9% increase in perspective, in the study by Merzlyakov et al.,³⁴ Cy3-TM_{FGFR3} peptide diffusivity increases ranging from 15% to 92% were evidenced with the introduction of PEG₃₀₀₀ polymer cushions. Our hypothesis is that the presence of the highly conformationally disordered PEG polymer cushion lower the aqueous phase viscosity on the substrate side of the supported bilayer giving rise to the increased diffusivity, relative to the untethered case where only a thin 0.5–2.0 nm thick water layer is separating the lipid bilayer from the solid surface.

Other studies have examined the diffusivity of smaller membrane-embedded peptides in lipid bilayers, for comparison, we have listed a few pertinent examples in Table 1. We note that in each case, the most important variable, lipid composition, differs significantly, as well as the peptide to lipid ratio. Lipid diffusivities, in general, are at least an order of magnitude larger ($5 - 0.1 \mu\text{m}^2/\text{s}$) than those of peptides and up to two orders of magnitude of higher than proteins, by virtue of the fact that their diffusion is confined to one leaflet of the bilayer with lower membrane embedded volumes. The toxin Gramicidin D shares this single leaflet characteristic, a cyclic decapeptide of well-characterized structure. Gramicidin D is shown to possess a diffusivity that is over 200 times larger than that of K₃A₄L₂A₇L₂A₃K₂-FITC.³⁵ Possibly the most relevant case is that of AKKL₁₈GKK-FITC, a 24-mer with highly similar structure studied by Gambin et al. in giant unilamellar vesicles (GUVs) at 1:1000 peptide to lipid molar ratios in SOPC.³⁶ In their work, the information concerning the characterization of the FITC labeling is limited, and it is unclear which amino group was

labeled (lysine ϵ -amino or N-terminus) as the diffusivity is greater than 18-fold larger than that of our peptide in polymer-cushioned supported bilayers. We note that the level of peptide loading is at least two orders of magnitude higher in our studies, leading to an increase in the peptide-peptide interactions within the bilayer. Furthermore, the use of the GUV system presumably precluded peptide-substrate interactions that would slow diffusion. Another relevant study was performed by Merzlyakov et al.,³² involving a rhodamine-labeled 33-mer based on the transmembrane domain of the FGF-receptor. Using planar supported bilayers at 1:2000 peptide to lipid ratio, the 33-mer exhibited a less than four-fold smaller diffusivity than for our 23-mer. This is not altogether unexpected. If we compare the two structures, our peptide length could be considered a “canonical” membrane-bound α -helix length, anchoring the hydrophobic region of the DOPC by lysines at the C- and N-termini. In our case the $A_4L_2A_7L_2A_3$ core forms a 2.7 nm long hydrophobic helical domain that is well-matched to the hydrophobic core of DOPC, a lipid bilayer structure experimentally determined to have a hydrophobic core thickness of ~ 2.72 nm when hydrated.³⁷ In contrast, the 33-mer has a significantly longer rhodamine-labeled 9-residue region protruding out from the postulated hydrophobic domain, also assumed helical in structural models supported by CD data that would conceivably add hydrodynamic drag to the C-terminus in the bilayer.

In our FRAP studies, no evidence was seen for larger multimeric peptide complexes with longer recovery times. To probe for the presence of substantial peptide-peptide interactions influencing peptide mobility, we examined the diffusivity of $K_3A_4L_2A_7L_2A_3K_2$ -FITC peptides within untethered and tether-supported lipid bilayers at two-fold lower peptide to lipid mass ratios (1:20). Although these FRAP measurements were carried out near the limits of obtaining adequate FITC signal-to-noise ratios in the CLSM (1:20 mass ratio $\approx$ 1:60 peptide to lipid mole ratio), the changes in diffusivity were not statistically significant, although significantly greater diffusivity was evidenced when polymer cushioning (tethered) was employed at these conditions. The increased diffusivity was likely due to a decrease in the viscosity of the lipid bilayers by decreasing the concentration of peptides. This is from two possible effects, a decreased number of peptide-peptide collisions and a lower number of crosslinked peptide anchors that result in fewer fixed obstacles in the diffusion path. For the untethered case, the diffusivity increase is somewhat less pronounced. By comparison, as the lipobead concentration of peptide decreased by half (molar ratio of peptide to lipid ranged from 1:30 to 1:60), the peptide diffusivity within lipid bilayers increased by $\sim 28.3\%$; over the same interval the diffusivity within lipid bilayers increased $\sim 17.9\%$ for the untethered case. The data is consistent with a scenario where a minor fraction of the peptides forms multimeric peptide complexes under the conditions studied, although the repulsion of the flanking lysine residues is designed to suppress this effect.

The further influence of the PEG₃₀₀₀ bilayer-to-substrate tethering layer versus a thin water layer was also evidenced in a small, yet statistically significant 6.4% increase in mobile fraction, α , in the 1:10 polymer cushioned case, shown in Figure 5. However, at the lower peptide to lipid ratios there was no significant difference in mobile fraction. There are a few factors that could be expected to affect peptide lateral mobility that directly influence the mobile fraction: 1) strong interactions with the substrate surface that lead to peptide immobility or “pinning”, 2) covalent tethering of labeled peptides in the polymer-cushioned case and 3) immobile regions in the bilayer due to peptide aggregation and 4) incomplete bilayer formation leading to discontinuous supported bilayer regions isolated from lateral diffusion (also consistent with immobilized, unfused peptide proteoliposomes). In the polymer-cushioned case, there is likely a trade-off between steric inhibition of peptide pinning by the cushion (increasing α and covalent anchoring (decreasing α)). However, we estimate that less than 2% of the total supported biomembrane area is anchored peptides, assuming that in the idealized upper-bound scenario where smooth microspheres are

completely covered with activated PEG₃₀₀₀-NHS ($R_F = 4.4$ nm; $A_{\text{PEG3000}} = 60.8$ nm²) that each connect to a single peptide in the biomembrane. However, three main factors may make this upper bound an overestimate; 1) the NHS-PEG-NHS reagent is not 100% pure and may contain a significant fraction of deactivated NHS as delivered from the manufacturer, 2) the silica-PEG-NHS is undergoing simultaneous hydrolytic deactivation during the tethering reaction and 3) the NHS to NH₂-peptide crosslinking reaction is highly peptide concentration dependent. Taken together, this could bring the level of the total supported biomembrane area that is anchored peptides down to below 1%, however this is above the threshold of crosslinking needed to form tether supported lipid bilayers. Judging from the essentially peptide concentration independent levels of the mobile fraction, α , shown in Figure 5, and the coverage, shown in Figure 4, we conclude that the levels of crosslinking are low enough so as to not significantly decrease the mobile fraction yet high enough to give good bilayer coverage around the silica microsphere. The high mobile fraction level and lateral mobility of untethered K₃A₄L₂A₇L₂A₃K₂-FITC in these tether-supported bilayers will provide a new mechanism to build laterally-mobile ligand displays based on these peptides, as has been done with peptide amphiphiles^{38, 39} and headgroup functionalized lipids in 2D systems.⁴⁰ In these systems, we envision fluorophore-trackable alpha-helices used as ligand anchors on the pPLB surface, with peptide localization changes due to interactions potentially serving a sensing function.

CONCLUSIONS

In this work, we have successfully synthesized a fluorescent alpha-helical peptide derivative, K₃A₄L₂A₇L₂A₃K₂-FITC at high purity and realized the reconstitution of peptide-anchor tether supported lipid bilayers by applying a directed-assembly approach based on liposome fusion. The novelty in this approach is the rational design and incorporation of the peptides into the lipid bilayers at the aqueous/lipid film interface, allowing for the control over the construction of supported membrane assemblies with peptide concentration and a built-in means to examine peptide localization and mobility.

The synthesized peptide complexes, exhibited a high alpha-helical content for stable spanning into lipid bilayers. An in-depth analysis of surface coverage was conducted based on randomly selected microspheres of each type (tethered and untethered case, molar ratio of peptide to lipid is 1:32, 1:48 and 1:64 respectively). The supported lipid bilayer coverage is slightly lower in the polymer-cushioned tethering case than untethered lipobeads. The fluidity measurements of fluorescent labeled peptides within lipid bilayers have been conducted via the confocal FRAP technique. The tethered peptide serves as an anchoring construct in polymer cushioned supported biomembranes, the unanchored K₃A₄L₂A₇L₂A₃K₂-FITC peptides within tether-supported lipid bilayers have a greater recovery and diffusivity than in the untethered control, where the lubricating “polymer cushion” layer offers a better mobility for the embedded peptides. By comparison, as the concentration of peptide decreased by half (molar mass ratio of peptide to lipid ranged from 1:30 to 1:60), the diffusivity of lipid bilayers increased by ~17.9%; over the same interval the diffusivity of lipid bilayers increased ~7.5% for the untethered case. Our results demonstrate that peptide-anchored supported lipid bilayers, exhibiting high lateral mobility, are well-suited for the purpose for investigations of native bilayer environments and as novel route for building ligand displays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by grants from the National Institutes of Health and the National Science foundation.

ABBREVIATIONS

FRAP	Fluorescence Recovery after photobleaching
pPLB	Peptide proteolipobead
ESI-MS	Electrospray Ionization Mass Spectrometry
Dde	(4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidine)ethyl
tBLM	tethered bilayer lipid membrane
NHS	N-hydroxysuccinimide
GUV	giant unilamellar vesicles
PEG	Poly(ethylene glycol)

References

1. Tanaka M, Sackmann E. Polymer-supported membranes as models of the cell surface. *Nature*. 2005; 437 (7059):656–63. [PubMed: 16193040]
2. Yu CH, Groves JT. Engineering supported membranes for cell biology. *Med Biol Eng Comput*. 2010; 48 (10):955–63. [PubMed: 20559751]
3. Bayerl TM, Bloom M. Physical properties of single phospholipid bilayers adsorbed to micro glass beads. A new vesicular model system studied by 2H-nuclear magnetic resonance. *Biophys J*. 1990; 58 (2):357–62. [PubMed: 2207243]
4. Johnson SJ, Bayerl TM, McDermott DC, Adam GW, Rennie AR, Thomas RK, Sackmann E. Structure of an adsorbed dimyristoylphosphatidylcholine bilayer measured with specular reflection of neutrons. *Biophys J*. 1991; 59 (2):289–94. [PubMed: 2009353]
5. Lambacher A, Fromherz P. Fluorescence interference-contrast microscopy on oxidized silicon using a monomolecular dye layer. *Appl Phys A*. 1996; A 63:207–216.
6. Tamm LK, McConnell HM. Supported phospholipid bilayers. *Biophys J*. 1985; 47 (1):105–13. [PubMed: 3978184]
7. Goennenwein S, Tanaka M, Hu B, Moroder L, Sackmann E. Functional incorporation of integrins into solid supported membranes on ultrathin films of cellulose: Impact on adhesion. *Biophysical Journal*. 2003; 85:646–655. [PubMed: 12829518]
8. Knoll W, Frank CW, Heibel C, Naumann R, Offenhausser A, Ruhe J, Schmidt EK, Shen WW, Sinner A. Functional tethered lipid bilayers. *Rev Mol Biotechnol*. 2000; (74):137–158.
9. Sackmann E, Tanaka M. Supported membranes on soft polymer cushions: fabrication, characterization and applications. *Trends Biotechnol*. 2000; 18 (2):58–64. [PubMed: 10652510]
10. Wagner ML, Tamm LK. Tethered polymer-supported planar lipid bilayers for reconstitution of integral membrane proteins: silane-polyethyleneglycol-lipid as a cushion and covalent linker. *Biophys J*. 2000; 79 (3):1400–14. [PubMed: 10969002]
11. Wagner ML, Tamm LK. Reconstituted syntaxin1a/SNAP25 interacts with negatively charged lipids as measured by lateral diffusion in planar supported bilayers. *Biophys J*. 2001; 81 (1):266–75. [PubMed: 11423412]
12. Cornell BAL, B-MV, King LGD, OP, BRLWJPR. A biosensor that uses ion-channel switches. *Nature*. 1997; 387:580–583. [PubMed: 9177344]
13. Schiller SM, Naumann R, Lovejoy K, Kunz H, Knoll W. Novel Archaea Analogue Thiolipids for Tethered Bilayer Lipid Membranes on Ultra Flat Gold Surfaces. *Angew Chem Int Ed*. 2003; 42:208–211.

14. Bunjes N, Schmidt E, Jonczyk A, Rippmann F, Beyer D, Ringsdorf H, Gräber P, Knoll WRN. Thiopeptide-Supported Lipid Layers on Solid Substrates. *Langmuir*. 1997
15. Buranda T, Huang J, Ramarao GV, Ista LK, Larson RS, Ward TL, Sklar LA, Lopez GP. Biomimetic Molecular Assemblies on Glass and Mesoporous Silica Microbeads for Biotechnology. *Langmuir*. 2003; 19 (5):1654–1663.
16. Troutier AL, Ladaviere C. An overview of lipid membrane supported by colloidal particles. *Adv Colloid Interface Sci*. 2007; 133 (1):1–21. [PubMed: 17397791]
17. Chemburu S, Fenton K, Lopez GP, Zeineldin R. Biomimetic silica microspheres in biosensing. *Molecules*. 2010; 15 (3):1932–57. [PubMed: 20336023]
18. Kumita JR, Smart OS, Woolley GA. Photo-control of helix content in a short peptide. *Proc Natl Acad Sci U S A*. 2000; 97 (8):3803–8. [PubMed: 10760254]
19. Tarasova NI, Seth R, Tarasov SG, Kosakowska-Cholody T, Hrycyna CA, Gottesman MM, Michejda CJ. Transmembrane inhibitors of P-glycoprotein, an ABC transporter. *J Med Chem*. 2005; 48 (11):3768–75. [PubMed: 15916428]
20. Rothe U, Aurich H. Lipid-Coated Particles—A New Approach to Fix Membrane-Bound Enzymes onto Carrier Surfaces. *Biotechnology and Applied Biochemistry*. 1989; 11:18–30. [PubMed: 2706088]
21. Schmidt EK, Liebermann T, Kreiter M, Jonczyk A, Naumann R, Offenhausser A, Neumann E, Kukol A, Maelicke A, Knoll W. Incorporation of the acetylcholine receptor dimer from *Torpedo californica* in a peptide supported lipid membrane investigated by surface plasmon and fluorescence spectroscopy. *Biosens Bioelectron*. 1998; 13 (6):585–91. [PubMed: 9828354]
22. Giess F, Friedrich MG, Heberle J, Naumann RL, Knoll W. The protein-tethered lipid bilayer: a novel mimic of the biological membrane. *Biophys J*. 2004; 87 (5):3213–20. [PubMed: 15339795]
23. Becucci L, Carbone MV, Biagiotti T, D'Amico M, Olivotto M, Guidelli R. Incorporation of the HERG potassium channel in a mercury supported lipid bilayer. *The Journal of Physical Chemistry B*. 2008; 112 (4):1315–1319. [PubMed: 18181608]
24. Becucci L, Martinuzzi S, Monetti E, Mercatelli R, Quercioli F, Battistel D, Guidelli R. Electrochemical impedance spectroscopy and fluorescence lifetime imaging of lipid mixtures self-assembled on mercury. *Soft Matter*. 2010; 6 (12):2733–2741.
25. Sharma MK, Gilchrist ML. Templated Assembly of Solid-Supported Membranes on Silica Microspheres using Bacteriorhodopsin Conjugates as Integrated Anchors. *Langmuir*. 2007; 23:7112–7123.
26. Stelzer, EHK.; Jonkman, JE. Resolution and Contrast in Confocal and Two-Photon Microscopy. In: Diaspro, A., editor. *Confocal and Two-Photon Microscopy: Foundations, Applications and Advances*. Wiley-Liss, Inc; New York: 2002. p. 101-125.
27. Klonis N, Rug M, Harper I, Wickham M, Cowman A, Tilley L. Fluorescence photobleaching analysis for the study of cellular dynamics. *Eur Biophys J*. 2002; 31 (1):36–51. [PubMed: 12046896]
28. Tsuji A, Ohnishi S. Restriction of the lateral motion of band 3 in the erythrocyte membrane by the cytoskeletal network: dependence on spectrin association state. *Biochemistry*. 1986; 25 (20):6133–9. [PubMed: 3790510]
29. Bechinger B. Membrane insertion and orientation of polyalanine peptides: a (15)N solid-state NMR spectroscopy investigation. *Biophys J*. 2001; 81 (4):2251–6. [PubMed: 11566795]
30. Percot A, Zhu XX, Lafleur M. Design and Characterization of Anchoring Amphiphilic Peptides and Their Interactions with Lipid Vesicles. *Biopolymers*. 1999; 50:647–655. [PubMed: 10508967]
31. Sugawara M, Resende JM, Moraes CM, Marquette A, Chich JF, Metz-Boutigue MH, Bechinger B. Membrane structure and interactions of human catestatin by multidimensional solution and solid-state NMR spectroscopy. *FASEB J*. 2010; 24 (6):1737–46. [PubMed: 20103720]
32. Merzlyakov M, Li E, Hristova K. Directed assembly of surface-supported bilayers with transmembrane helices. *Langmuir*. 2006; 22 (3):1247–53. [PubMed: 16430290]
33. Mackenzie KR. Folding and Stability of α -Helical Integral Membrane Proteins. *Chemical Reviews*. 2006; A:1–41.

34. Merzlyakov M, Li E, Gitsov I, Hristova K. Surface-supported bilayers with transmembrane proteins: role of the polymer cushion revisited. *Langmuir*. 2006; 22 (24):10145–51. [PubMed: 17107013]
35. Wu ES, Jacobson K, Szoka F, Portis A Jr. Lateral diffusion of a hydrophobic peptide, N-4-nitrobenz-2-oxa-1, 3-diazole gramicidin S, in phospholipid multibilayers. *Biochemistry*. 1978; 17 (25):5543–50. [PubMed: 83159]
36. Gambin Y, Lopez-Esparza R, Reffay MES, Gov NS, Genest M, Hodges RS, Urbach W. Lateral mobility of proteins in liquid membranes revisited. *Proceedings of the National Academy of Science*. 2006; 103:2098–2102.
37. Pan J, Tieleman DP, Nagle JF, Kucerka N, Tristram-Nagle S. Alamethicin in lipid bilayers: combined use of X-ray scattering and MD simulations. *Biochim Biophys Acta*. 2009; 1788(6): 1387–97. [PubMed: 19248763]
38. Ananthanarayanan B, Little L, Schaffer DV, Healy KE, Tirrell M. Neural stem cell adhesion and proliferation on phospholipid bilayers functionalized with RGD peptides. *Biomaterials*. 2010; 31 (33):8706–15. [PubMed: 20728935]
39. Kokkoli E, Mardilovich A, Wedekind A, Rexiesen EL, Garg A, Craig JA. Self-assembly and applications of biomimetic and bioactive peptide-amphiphiles. *Soft Matter*. 2010; 2:1015–1024.
40. Garcia AS, Dellatore SM, Messersmith PB, Miller WM. Effects of supported lipid monolayer fluidity on the adhesion of hematopoietic progenitor cell lines to fibronectin-derived peptide ligands for alpha5beta1 and alpha4beta1 integrins. *Langmuir*. 2009; 25 (5):2994–3002. [PubMed: 19437769]

Figure 1.

Flowchart and schematic diagram of peptide proteolipobead construction. The lipid and $K_3A_4L_2A_7L_2A_3K_2$ -FITC are mixed in solvent and dried to give dried lipid film followed by probe sonication to give peptide proteoliposomes, and then fusion and tethering onto PEG₃₀₀₀-NHS activated 4.7 μ m silica microspheres. The rightmost top picture shows the idealized schematic structure of a **(1) polymer cushion tethered peptide proteolipobead**, with tethered and untethered peptides depicted. Below on the right is the schematic of a **(2) control untethered peptide proteolipobead**.

Figure 2.

Electrospray Ionization-Mass spectrometry of the conjugated peptide $K_3A_4L_2A_7L_2A_3K_2$ -FITC. The main species (*) obtained exhibited m/z value of 392.2 m/z, with six positive charges and a calculated molecular weight of 2349.6 g/mol, consistent with the species $K_3A_4L_2A_7L_2A_3K_2$ -FITC. The unlabeled peptide is evidenced at 257.4 m/z in the mass spectrum, denoted by (**).

Figure 3.

Circular Dichroism spectra of the synthesized peptide in the liposomes. The CD spectrum from the FITC-labeled peptide at a concentration of 0.2mg/ml in buffer is given by the dotted line. The solid line is the simulation using CD basis sets and gives an ensemble average alpha-helicity of 80.7% with the remaining 19.3% as random coil. (peptide-FITC: POPC=1:30: RMSD diff = 0.34).

Figure 4. Representative 3D Reconstructions of Confocal Image Stacks of $4.7\ \mu\text{m}$ $\text{K}_3\text{A}_4\text{L}_2\text{A}_7\text{L}_2\text{A}_3\text{K}_2$ -FITC proteolipobeads (peptide:lipid=1:32 in molar ratio), excitation wavelength 488nm, Detection window 500–615 nm; 3D reconstruction views of control untethered peptide proteolipobeads (A), and polymer cushion tethered peptide proteolipobeads (B). The white arrows indicate representative visible inhomogeneities. Panel (C) is the lipid coverage fraction metric measured from equatorial lipobead z slices ($N = 40$); Panel (D) is the average number of aggregates per pPLB assembly ($N = 20$); Values are given $\pm$ standard error ($\sigma/3N$). (Diagonal (blue) patterned bars: control untethered peptide proteolipobeads). Dotted (red) patterned bars: polymer cushion tethered peptide proteolipobeads),

Figure 5.

FRAP data Analysis: Mean value of Diffusion coefficient and recovery fraction. Sample size is $N = 40$, and the mass ratio of peptide to lipid is 1:10, 1:15 and 1:20 respectively. Diagonal pattern (left blue bars): control untethered peptide proteolipobeads. Dotted pattern (right red bars): polymer cushion tethered peptide proteolipobeads. Values are given $\pm$ standard error ($\sigma/3N$).

Table 1

Comparison of Transmembrane Peptide and Lipid Diffusion

Peptide + Major Lipid	D ($\mu\text{m}^2/\text{s}$)
AKKL ₁₈ GKK-FITC (GUVs 99%SOPC 1:1000) ³⁶	0.31±0.02
FGFR TM Peptide in supported membrane, (99%POPC 1:2000, 33mer) ³²	0.0055±0.0001
Gramicidin S (Egg PC, cyclic decamer) ³⁵	3.6±0.6
K ₃ A ₄ L ₂ A ₇ L ₂ A ₃ K ₂ -FITC in untethered supported membrane (peptide: DOPC=1:20 (mass))	0.0190±0.0005
K ₃ A ₄ L ₂ A ₇ L ₂ A ₃ K ₂ -FITC tethered solid supported membrane (peptide: DOPC=1:20(mass))	0.0245±0.0007