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Biodegradable, Tethered Lipid Bilayer–Microsphere Systems with Membrane-Integrated α -Helical Peptide Anchors

Eric S. Fried[†], Joshua Luchan[‡], and M. Lane Gilchrist^{*,†,‡}

[†]Department of Chemical Engineering, The City College of the City University of New York, 140th Street and Convent Avenue, New York, New York 10031, United States

[‡]Department of Biomedical Engineering, The City College of the City University of New York, 140th Street and Convent Avenue, New York, New York 10031, United States

Abstract

Supported lipid bilayers (SLBs) are ideally suited for the study of biomembrane–biomembrane interactions and for the biomimicry of cell-to-cell communication, allowing for surface ligand displays that contain laterally mobile elements. However, the SLB paradigm does not include three-dimensionality and bio-compatibility. As a way to bypass these limitations, we have developed a biodegradable form of microsphere SLBs, also known as proteolipobeads (PLBs), using PLGA microspheres. Microspheres were synthesized using solvent evaporation and size selected with fluorescence activated cell sorting (FACS). Biomembranes were covalently tethered upon fusion to microsphere supports via short-chain PEG spacers connecting membrane-integrated α -helical peptides and the microsphere surface, affecting membrane diffusivity and mobility as indicated by confocal FRAP analysis. Membrane heterogeneities, which are attributed to PLGA hydrophobicity and rough surface topography, are curtailed by the addition of PEG tethers. This method allows for the presentation of tethered, laterally mobile biomembranes in three dimensions with functionally embedded attachment peptides for mobile ligand displays.

*Corresponding Author gilchrist@ccny.cuny.edu; Ph 212-650-6664 (M.L.G.).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.6b00008. An extended description of the methods, including the materials, peptide synthesis procedures, PLGA substrate preparation, and details regarding imaging measurements (PDF)

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INTRODUCTION

Supported lipid bilayers (SLBs) are model biomembrane platforms that have found extensive application in biophysical, biomimetic, tissue engineering, and sensor design studies due to their increased stability and ease of modification over free-standing membranes. SLBs are formed mainly from the adsorption, deformation, and rupture of small unilamellar vesicle (SUV) liposomes to the solid interface. Silica, mica, and quartz surfaces are typical biomembrane supports due to their hydrophilicity and smooth topographies.¹⁻⁴ In the context of tissue engineering and the investigation of biomembrane–biomembrane cellular interactions, SLBs have recently been featured in studies designed for eliciting responses in live cells via cell membrane–SLB adhesion and subsequent signal transduction.⁵⁻⁸ To be applied toward viable regenerative approaches involving cellular remodeling of the microenvironment, it is required that the typical solid supports used be replaced by biocompatible substrates. The beginnings of this transition have featured lipid bilayers supported by polysaccharides,⁹ biocompatible polyelectrolytes, polymers, and hydrogels.⁹⁻¹¹

Poly(lactic-*co*-glycolic acid) (PLGA) is a commonly featured polymeric material for both tissue engineering and drug delivery due to its well-characterized biodegradation, commercial availability, and FDA approval.¹² Biomembranes supported by PLGA substrates have appeared in nanoparticle drug delivery platforms as well.^{13,14} To our knowledge, this ideal substrate material has not been applied to studies involving on biomembrane–biomembrane interactions and biomimetic membranes. Moreover, the ease of PLGA microsphere synthesis strategies^{15,16} permits the fabrication of cell-sized PLGA-based spherical SLBs. These constructs would be feasible for 3D biomembrane-to-biomembrane interactions in the “phantom cell” context,¹⁷ furthering the biomimetic nature of tissue engineering constructs.

Here, we present the design and synthesis of biomembranes supported by PLGA microspheres with membrane-integrated α -helical peptide anchors, termed proteolipobeads (PLBs). These assemblies feature short-chain poly(ethylene glycol) (PEG) tethers to provide nucleation for membrane fusion at peptide–PEG anchoring sites and to impart mechanical stability of fused membranes. PLGA particles were first characterized by their size distribution, and particle size selection was performed with fluorescence-activated cell sorting (FACS). Tethered and control PLBs were assessed for membrane coverage with confocal laser scanning microscopy (CLSM); peptide diffusivity and mobility were measured by biomembrane fluorescence recovery after photobleaching (FRAP).

EXPERIMENTAL SECTION

Materials—1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL). K₃A₄L₂A₇L₂A₃K-Fmoc-Lys(Dde)-OH (α -helical peptide) was custom synthesized by and purchased from Biobasic Inc. (Markham, Ontario, Canada). Fluorescein isothiocyanate (FITC), chloroform, *N,N*-dimethyl-formamide (DMF), methyl *tert*-butyl ether (MtBE), bis-*N*-succinimidyl(nonaethylene glycol) ester (BS(PEG)₉), anhydrous ethylenediamine, and Fisherbrand 40 μ m cell strainers were obtained from ThermoFisher Scientific Corp. (Chicago, IL). Trifluoroacetic acid (TFA, 95%), hydrazine,

N,N-diisopropylethylamine (DIEA), piperidine, and Resomer RG504 H Poly(D,L-lactide-*co*-glycolide) (PLGA) were purchased from Sigma-Aldrich (St. Louis, MO). Poly(vinyl alcohol) (PVA) was purchased from Acros Organics. FITC-PEG_{5k}-NHS (MW 5000) was obtained from Nanocs, Inc. (New York, NY). Nonporous SiO₂ (silica) microspheres of 5.09 μ m average diameter were purchased from Bangs Laboratories, Inc. (Fishers, IN).

PLGA Microsphere Synthesis and Surface Modification—PLGA microspheres were synthesized using an emulsion-evaporation technique.^{15,16} Dry PLGA was dissolved at 1 mg/mL in chloroform and added dropwise to 50 mL of 0.5% PVA stirring at 550 rpm using a magnetic stirring plate. The organic phase was allowed to evaporate overnight. The resulting microsphere suspension was washed twice in diH₂O and then passed three times through 40 μ m cell strainers. Amine functional groups were generated on particle surfaces by aminolysis with ethylenediamine.¹⁸ The filtered PLGA particle suspension was added to aqueous ethylenediamine to a final concentration of 50 mM and incubated for 45 min, followed by rinsing in diH₂O. Successful aminolysis was separately assessed by attachment of FITC-PEG₅₀₀₀-NHS incubated at 70 μ g/mL for 45 min.

Microsphere Size Sorting—Flow cytometry/fluorescence-assisted cell sorting (FACS) was performed to characterize unmodified PLGA particles for their size distribution and to sort microspheres by size using a BD FACS Aria II cell sorter. Particle size distribution was observed with a forward scatter histogram. Three gate regions were placed within this histogram to mark three subpopulations of microsphere size. These gated regions were sorted into separate tubes.

PLGA PLB Construction and Characterization—A lipid/peptide mixture containing 96% DOPC and 4% FITC-labeled peptide by mole dissolved in chloroform was dried under vacuum overnight and hydrated at 2 mg/mL in PBS buffer. The resulting multilamellar vesicle (MLV) suspension was then probe sonicated in an ice bath using a BioLogics Inc. 150 V/T ultrasonic homogenizer for 15 min, forming small unilamellar vesicle liposomes (SUVs). Separately, 250 mM BS(PEG)₉ in DMSO was added to aminolyzed PLGA particles in aqueous suspension to a final PEG concentration of 30 mg/mL. The tethering approach utilizes BS(PEG)₉ as a homobifunctional amine cross-linker, connecting amine groups present on PLGA surfaces to those present on the peptide lysines at both the N- and C-termini. A control sample was prepared without particle exposure to BS(PEG)₉. The suspension was briefly vortexed and then incubated for 15 min followed by a brief rinsing. Finally, NHS-PEG and control microspheres were added to SUV suspensions, briefly vortexed, and incubated for 1 h, followed by rinsing of the excess liposomes to yield biodegradable, tethered PLGA PLBs. As PLB controls, peptide-liposomes were also fused to bare 5 μ m diameter silica microspheres for comparison to PLGA PLBs. All PLBs were imaged with confocal microscopy and peptide lateral mobility within PLGA PLB membranes was characterized using confocal FRAP.

RESULTS AND DISCUSSION

As an FDA-approved medical material with widespread use in tissue engineering, PLGA was selected as a solid microsphere support to build biodegradable PLBs. PLGA

microspheres were synthesized through emulsion evaporation. Post synthesis, the microspheres were imaged in DIC to assess the resulting size distribution (Figure 2). The average microsphere diameter is $11.4 \pm 1.66 \mu\text{m}$ with a standard deviation of $20.9 \mu\text{m}$. This population of microspheres is highly polydisperse, which for the present application is acceptable. However, an effective filtration or sorting strategy would be necessary for applications involving live tissue or cells, as the microsphere diameter is known to influence cellular behavior and can promote particle internalization by cells.^{19,20} Using FACS as a proof-of-concept, PLGA microspheres were sorted into three size populations based on peaks observed in forward scatter profile. The first population gated (Figure 2, Sort ROI A) contained the smallest diameter particles, which made up the most populated size group. This indicates that the particle synthesis protocol used is skewed toward smaller ($2\text{--}5 \mu\text{m}$) diameter particles that are likely too small for cell–cell-like interactions with living cells due to the potential for particle internalization by cells. The larger diameter sort groups (Sort ROIs B and C) would satisfy particle size constraints for cellular interactions. Hence, a modified microsphere synthesis protocol that favors larger ($>15 \mu\text{m}$) particles would be advantageous. In order to generate functional amines groups on the microsphere surface, PLGA microspheres were aminolyzed using 50 mM ethylenediamine. Successful generation of surface amines was assessed by attachment of FITC-PEG₅₀₀₀-NHS versus an unmodified control. Using confocal *z*-stack projections displaying sum fluorescence intensity and correcting for variation in particle size, aminolyzed spheres exhibited 2.26-fold surface fluorescence intensity over nonspecific binding of FITC-PEG₅₀₀₀-NHS to unmodified controls ($6802.5 \pm 352.66 \text{ AU}/\mu\text{m}^2$ vs $3013.4 \pm 525.73 \text{ AU}/\mu\text{m}^2$), indicating sufficient surface amine presentation.

Following synthesis, PLGA microspheres were aminolyzed by ethylenediamine exposure, decorated with BS(PEG)₉ and fused with peptide liposomes (Figure 1). This resulted in biodegradable PLB formation with polymer tethers and integral membrane peptides, which have straightforward application in tissue engineering research involving biotic–abiotic interfaces. Imaging by confocal laser scanning microscopy shows biomembrane coverage of microspheres as indicated by FITC signal (Figure 3). Figure 3, panel A, is a confocal 3D reconstruction of an assortment of PLGA PLBs of various sizes, ranging from 5 to $14 \mu\text{m}$ in diameter. Continuous FITC signal from embedded peptides is present throughout the microsphere surfaces, indicative of supported bilayer formation. Larger spheres ($>10 \mu\text{m}$) display a loss of signal intensity above equatorial regions due to focal-length-dependent optical spherical aberration and refractive index mismatch of the water surrounding the PLGA microsphere in confocal 3D reconstructions (resulting in low signal highlighted in blue). PLBs below this size threshold were used to calculate the approximate coverage of homogeneous bilayer by measurement at equatorial planes. From $N=10$ tethered and control PLBs, PEG-tethered PLBs were measured to be $94.6 \pm 1.61\%$ uniform in peptide/membrane coverage, while the nontethered control was $82.0 \pm 3.27\%$ uniform ($p=0.003$).

Figure 3, panel B, is a gallery of hemispherical *z*-projections of individual PLGA PLBs. These same *z*-projections are shown false colored in panel C of Figure 3 to highlight differences in fluorescence intensity. The higher intensity heterogeneities in CLSM allow us to discern between larger defects seen in CLSM that are consistent with adsorbed vesicles that did not fuse. In the leftmost and center projections we see clear evidence for nonoptimal

mixed vesicle absorption, although in these cases less than ~10% of the surface is covered by these imperfections. In our PLB systems, two factors degrade resolution: (1) out-of-focus light from the high concentrations of fluorophores in adjacent *z*-sections and (2) refractive index mismatch of the bead substrates and the surrounding medium that gives rise to *z*-dependent optical spherical aberration. These factors also impede the reliable use of 3D deconvolution, especially in PLBs of different sizes. Nevertheless, the application of the CLSM to PLBs has allowed us to assess supported biomembrane quality.

To compare the interfacial topography of PLGA-supported PLBs to traditional silica-supported systems, measurement of the perceived membrane thickness in equatorial *z*-section confocal images with full width at half-maximum (fwhm) calculation is a useful tool. Equatorial *z*-section CLSM images used for these measurements are displayed in Figure 3, panel D. Though the diffraction-limited lens of optical microscopy does not provide authentic nanoscale distance measurements, comparison of fwhm values can reveal substantial information in lieu of direct surface roughness characterization. In this way, membrane contouring to increased particle surface roughness would increase the volume from which fluorophores emanate photons, producing a perceivably thicker membrane under the diffraction limited resolution of optical microscopy. For *N* = 10 constructs, the fwhm membrane thickness measured at equatorial planes was found to be $0.576 \pm 0.07 \mu\text{m}$ for PLGA PLBs and $0.333 \pm 0.02 \mu\text{m}$ for silica PLBs ($p = 0.002$). This measurement is consistent with increased surface roughness of PLGA relative to silica, with the more complex topography giving rise to a wider surface emission region in an optical section.

Control over membrane diffusive characteristics is highly significant in tissue engineering approaches that exploit cellular signaling pathways involving mobile ligands such as *N*-cadherin. By implementing a dual membrane spacing/tethering strategy, laterally mobile membranes can be formed with control over peptide and membrane diffusivity. Using a short homobifunctional cross-linker BS(PEG)₉ at a high reaction concentration (30 mg/mL), tethered PLBs were formed with the diffusivity and mobile fraction of peptide-FITC measurable by FRAP. The effective diffusion coefficients, D_{eff} , of BS(PEG)₉-tethered PLBs and nontethered controls were measured to be 0.010 ± 0.001 and $0.017 \pm 0.003 \mu\text{m}^2/\text{s}$, respectively ($p = 0.045$). In addition, mobile fractions of the tethered and nontethered samples were found to be 0.63 ± 0.07 and 0.74 ± 0.04 , respectively ($p = 0.22$).

Though the D_{eff} values attained are markedly low when compared to lipid or peptide diffusivities found in GUVs and giant plasma membrane vesicles (GPMVs), it has been previously confirmed that diffusion in model membrane vesicles is more than twice that of solid-supported biomembranes.²¹ Moreover, the peptide diffusivities measured here are in close agreement with identical membranes tethered to silica microspheres with PEG₂₀₀₀²² and are in the same order of magnitude as α -helical peptide diffusion in other supported membrane systems.²³ A significant result here are low mobile fractions of biodegradable PLBs, and even more striking is the effect of BS(PEG)₉ tethering relative to the nontethered control. When compared with our previous PEG₂₀₀₀-silica tethered PLBs and bare silica controls, the mobile fractions are reduced by a significant fraction. This is to be expected, as the well-characterized smoothness and high degree of hydrophilicity of silica substrates lead to self-healing SLBs with lipid mobile fractions near unity. By comparison, the complex and

heterogeneous PLGA polymer interface likely contains significant levels of liposome-adsorbent hydrophobic regions that would lead to loss of bilayer structural integrity and decrease in mobile fraction. This is coupled with a higher surface roughness at the nanoscale that would presumably lead to isolated bilayer regions that are not diffusively linked to the reservoir of FITC-labeled peptides that would lead to fluorescence recovery. Control PLGA PLBs had considerably higher diffusion and somewhat higher mobile fractions than tethered PLBs, yet the tethered constructs presented significantly more homogeneous membrane coverage of PLGA particles. It is apparent that tethering is necessary with PLGA substrates to form uniform membranes despite sacrificing some degree of lipid/peptide mobility and diffusivity.

Using PLGA in place of traditional glass or silica membrane supports confounds the classic adsorption–deformation–rupture–fusion scheme of SLB formation.²⁴ We acknowledge three variables that can dictate the extent of liposome fusion and lipid/peptide mobility as measured by FRAP: (1) intact SUV liposomes that were tethered to the microsphere interface without fusing, (2) overtethering that immobilizes a significant population of peptides, and (3) a rough PLGA interface topography that interferes with diffusive membrane transport.¹⁵ Though the data here suggest that lowered diffusivity of tethered membranes might be attributed to overtethering, the material effects and roughness of PLGA must also be considered.^{15,16,25}

The inherent distinctions between PLGA surfaces and prevalent lipid bilayer supports can affect the efficacy of liposome fusion. PLGA's hydrophobic nature, its known surface roughness, and the surface modification by aminolysis performed here all may impact liposome adsorption and rupture. It has been shown that titration of poly(dimethylsiloxane) (PDMS) surface hydrophobicity by plasma oxidation permits vesicle fusion once the interface becomes sufficiently hydrophilic.²⁶ Moreover, Bershteyn et al. found that DOPC liposomes do not rupture and fuse to the surface of bare PLGA nanoparticles as they do on silica particles,¹⁴ where hydrophobicity is the characteristic interfacial difference. Our treatment of PLGA interfaces with short PEG chains may improve hydrophilicity in addition to their functioning as anchoring sites, based on the homogeneity of tethered PLBs relative to the nontethered control.

Interfacial roughness is also known to affect liposome fusion, where depending on the substrate used, there exists a threshold roughness above which vesicle adsorption without rupture and fusion is promoted.^{27,28} This is likely due to an inhibition of rupture propagation. The surface roughness of PLGA microspheres prepared by solvent evaporation has been previously measured to be approximately 12 nm or below²⁹ which reaches the limit suggested Duarte and Raposo. Moreover, the fragmentation of polymer chains during aminolysis treatment likely increases this value and thereby decreases the probability of vesicle fusion after adsorption.¹⁸ Conversely, classic biomembrane supports such as silica have surface roughnesses below 1 nm and exhibit high mobile fractions and self-healing due to the 1–2 nm water cushion.³⁰

It is possible that particle surface passivation would be improved by decoration with larger MW PEG chains in place of BS(PEG)₉. However, a higher density PEG attachment is

attainable with smaller chain lengths by the significant decrease in hydrodynamic polymer radius. A higher density of PEG tethers allows for increased PEG–peptide conjugation and membrane tethering. For these constructs to be viable with regard to cellular adhesion and the forces involved, mechanical stability of PLB membranes is essential.

CONCLUSIONS

We have demonstrated successful fabrication of laterally mobile lipid bilayers with integrated α -helical peptides covalently tethered to biodegradable PLGA microspheres. The diffusion coefficient measured for the peptide matches that of analogous systems involving hydrophilic substrates while lower mobile fractions arise from high amount of peptide anchoring and the roughness of PLGA surfaces. In this instance we observe a compromise between mobile fraction and extent of liposome–microsphere fusion due to the need for sufficient nucleation sites to initiate liposome rupture and supported bilayer formation. It would be advantageous to modify the PLGA particle synthesis to skew the size distribution toward 20–30 μm spheres. Furthermore, utilizing larger PEG chain tethers would better passivate PLGA hydrophobicity and override mobile fraction limitations due to surface roughness and polymer interface heterogeneity. Nonetheless, these structures are suitable for application in tissue engineering experiments in which cells can adhere to and receive directions from mobile ligand displays on PLB surfaces in biomimetic cell-to-cell signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FRAP	fluorescence recovery after photobleaching
PLGA	poly(lactic- <i>co</i> -glycolic acid)

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Figure 1.

Schematic flowchart of biodegradable PLB formation. Peptide-FITC/DOPC films (1:25 peptide:lipid by mole) mixed in chloroform and vacuum-dried are hydrated in PBS and probe sonicated to produce small unilamellar peptide liposomes. Simultaneously, dry PLGA is dissolved in chloroform and dispersed in aqueous PVA with overnight stirring to produce microspheres. Surface amine groups are then generated by treatment with ethylenediamine, which are then conjugated with BS(PEG)₉ homobifunctional cross-linker. The final PLBs are formed by simultaneous liposome fusion and peptide-BS(PEG)₉ conjugation.

Figure 2.

(top inset) Differential image contrast (DIC) image of PLGA microspheres after synthesis. Defects in synthesis such as internal void pockets are noticeable. (top) The raw size distribution is highly skewed to 2–5 μm diameter particles, with smaller particles going undetected by imaging. The bottom left panel is a 2D cytofluorogram of unsorted PLGA particles. The sort ROIs indicate the gating for FACS sorting to yield the three particle size subsets with the particle size histograms shown in the bottom right panels. Scale bar: 20 μm .

Figure 3.

3D imagery of PLGA PLBs. Panel A is a confocal 3D reconstruction of an assortment of PLGA PLBs of various sizes. Continuous FITC signal is present throughout the microsphere surfaces, indicative of supported bilayer formation. Equatorial fluorescence in microspheres above $\sim 10\ \mu\text{m}$ in diameter is distorted due to spherical aberration and refractive index mismatch, resulting in low signal highlighted in blue. Panel B is a gallery of hemispherical CLSM z-projections of individual PLGA PLBs. In the bottom panel C the z-projections are

presented as false color images. Panel D is a gallery of equatorial z sections from the PLBs in panels B and C. Scale bars in the galleries indicate $3\ \mu\text{m}$.

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