Published in final edited form as:

Soft Matter. 2008 ; 4(9): 1787–1791. doi:10.1039/b804933e.

Polymer-supported lipid shells, onions, and flowers

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Abstract

Phospholipid-enveloped biodegradable polymer microparticles and nanoparticles synthesized by an emulsion/solvent evaporation process were characterized by confocal and cryoelectron microscopies to show that the lipid envelope exhibits two-dimensional fluidity and can be configured into ‘shell’, ‘onion’, or ‘flower’ nanostructures, depending on the quantity and composition of lipids employed in the synthesis.

Phospholipid assemblies have been used to surround solid particles for studies of membrane interactions, biodegradable polymers for drug delivery, and live bacterial cells for protection against harsh surrounding environments^{1,2}. We are particularly interested in ‘lipid-enveloped’ polymer nanoparticles with a biodegradable solid polymer core, for potential applications in drug delivery or as model supported membranes for the study of host cell-pathogen interactions. Lipid-coated microparticles and nanoparticles are typically prepared via fusion of preformed liposomes with solid polymer or silica particle supports^{3–9}. An alternative approach is to fabricate polymer particles via an emulsion/solvent evaporation technique, using lipids as surfactants for particle synthesis. This strategy has been utilized for fabrication of lipid-containing biodegradable polymer particles such as poly(L-lactide) or poly(D,L-lactide-co-glycolide) (PLGA) for drug delivery^{10–16}. Enrichment of phospholipids at the surface of PLGA microparticles formed by emulsion approaches has been demonstrated via X-ray photoelectron spectroscopy and zeta potential analysis^{11–13}, but the lipid organization at the surfaces of such particles has only been examined at the level of light microscopy¹⁰. In this study, we investigated the rich variety of morphologies and nanostructures accessible by self-assembly of phospholipids and other biological membrane components at particle surfaces during emulsion-based particle synthesis. We used cryogenic transmission electron microscopy (cryo-TEM) to directly visualize the surface organization of lipids on these

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[†]Electronic Supplementary Information (ESI) available: Cryo-TEM micrographs showing bilayer-coated silica nanoparticles; bare polymer nanoparticles incubated with liposomes; abrogation of “onion” and “flower” formation at high lipid concentration by incorporation of negatively-charged DOPG; and preservation of “onion” and “flower” nanostructures by freeze-drying.

particles, because the rapid freezing of a thin film of aqueous sample leads to formation of vitreous ice, avoiding the volume change of crystalline ice that normally damages frozen, hydrated biological specimens^{17–19}. We found that dramatically different structures can be created by varying lipid concentration and composition.

As a first step toward the creation of polymer particles with surface lipid layers mimicking the composition of lipid-enveloped pathogens, we compared the self-assembly of different components of biological membranes (or synthetic analogs) at the surfaces of PLGA particles. We employed an emulsion/solvent evaporation approach to fabricate lipid-enveloped polymer microparticles and nanoparticles (Figure 1a): Polymer (50:50 wt:wt LA:GA, 46 kDa, Lakeshore Biomaterials, Birmingham, Alabama) and lipid (Avanti Polar Lipids, Alabaster, Alabama) were co-dissolved in 5 mL dichloromethane (DCM, Mallinckrodt Baker, Phillipsburg, New Jersey). The amount of phospholipid was fixed at 4.7 μ moles, for a polymer:phospholipid weight ratio of approximately 25:1. This organic solution was emulsified into 40 mL of deionized water for 6 minutes at 17,500 RPM using an Ika Ultra Turrax T25 Basic homogenizer at 25°C. The resulting emulsion was magnetically stirred for 12 hours at 25°C in a fume hood to evaporate the dichloromethane and form solid particles. The polydisperse particles formed in this synthesis were subsequently separated by centrifugation for 5 min at 2,000 RCF into cell-sized ($1.9 \pm 0.9 \mu$ m diameter, Figure 1b) and virus-sized (116 ± 35 nm mean diameter, Figure 1c) populations. Sizes were determined using a JEOL 6320 Field-Emission High-Resolution SEM after vacuum-drying of particles onto silicon and coating with 100 Å gold.

The lipid distribution in microparticles formed by this process was first examined by confocal microscopy. In line with prior studies, PLGA microparticles formed in the presence of 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Figure 2a) or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, not shown), where 1 mole % of the lipid was labeled on the headgroup with a rhodamine tag, exhibited a clear enrichment of lipid at the particle surfaces. Likewise, PLGA particles formed in the presence of a 99:1 mol:mol mixture of DMPC and biotin-PEG-DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) conjugated to a 2 kDa biotin-terminated poly(ethylene glycol) (PEG) linker) showed surface accessibility of the biotin label when stained post-synthesis with fluorescent streptavidin (Figure 2a). However, phospholipid surface segregation in PLGA particles was dependent on the composition of the external aqueous phase during particle synthesis; if the same particles were formed in a salt-containing buffer (150 mM phosphate buffered saline (PBS), pH 7.4) instead of deionized water, no evidence for enrichment of lipids labeled with either rhodamine (Figure 2b) or the smaller fluorescent tag N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD, not shown) at the surfaces of PLGA particles was found. In an external phase diluted to one-tenth the salt content (15 mM PBS), enrichment of rhodamine-lipid at the particle surfaces was again observed, suggesting that surface-segregation of these anionic labeled phospholipids was inhibited at high ionic strength. This was an unexpected finding as prior theoretical and experimental studies have shown that lipid adsorption at oil/water interfaces typically increases with increasing ionic strength, due to decreased ionic repulsion between charged lipid headgroups^{20, 21}. Although we cannot definitively show that unlabeled lipids exhibit similar trends in surface enrichment behavior by microscopy, because lipids with two entirely different fluorophore tags exhibit the same salt-dependent surface segregation behavior, these data suggest that for charged lipids (e.g., DOPG), surface enrichment may be sensitive to ionic strength. Next, particles were formed using a lipid membrane composition mimicking the envelope of the human immunodeficiency virus HIV-1 : 9 mole % DOPC, 9 mole% 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), 20 mole% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 18 mole% sphingomyelin, and 45 mole % cholesterol (same total molar quantity of phospholipid as before)^{22, 23}. These particles were labeled with 1 mole % rhodamine-conjugated DOPC or with cholesterol conjugated to the fluorescent NBD. While

DOPC-rhodamine localized to HIV-membrane-coated particle surfaces (Figure 2c), cholesterol-NBD was distributed throughout the particle bulk (Figure 2d). This result implies that, unlike liposomes, for which the presence of water on both sides of the lipid membrane favors the incorporation of added cholesterol in bilayers at high levels, cholesterol in lipid-enveloped particles can readily partition into the moderately hydrophobic polymer core and does not preferentially reside within the surface lipid layer. A qualitatively identical lack of surface segregation was obtained with PLGA particles containing lipid-like tracer molecules based on carbocyanine, which have acyl chain tails like phospholipids but less polar headgroups (data not shown). Hence, surface segregation and formation of a lipid shell does not occur with all lipid membrane components, nor in all conditions.

In order to truly mimic the properties of biological membranes, phospholipids assembled at particle surfaces should exhibit two-dimensional fluidity in the surface plane. Lipid mobility in supported membranes as well as in large unilamellar liposomes has been demonstrated using techniques such as fluorescence recovery after photobleaching (FRAP) ^{24–32}. We tested the mobility of surface-localized lipids by analyzing FRAP for particles between 3 and 8 microns in diameter with lipid layers labeled with 20 mole% NBD-DOPC (Figure 2e). To prevent Brownian motion during imaging, particles suspended in water were mixed at a 1:1 volume ratio with an aqueous alginate solution (2% wt/vol alginate in water) and mounted on a slide using a coverslip sprayed with CaCl₂ solution (200 mM in water) to induce gelation of the alginate prior to imaging on an upright Zeiss LSM 510 confocal laser scanning microscope. A wedge-shaped region of surface lipids on each particle was bleached with the full intensity of a 5.3 mW argon laser emitting at 488 nm, followed by imaging at 0.4% maximum laser intensity. Areas bleached by the high-intensity beam regained a fluorescence intensity comparable to the rest of the particle within ~5 s of photobleaching, indicating that the majority of the surface-localized phospholipids are mobile and rapidly diffuse over the surface of lipid-enveloped microparticles. Fitting of fluorescence recovery data such as shown in Figure 2e to an exponential model by a least-squares fit ^{33–36} gave an estimated diffusion coefficient of 0.4 $\mu\text{m}^2 \text{sec}^{-1}$, near the expected diffusion coefficients for supported lipid bilayers (~2 $\mu\text{m}^2/\text{s}$) ^{37–39}. Qualitatively similar results were obtained in 14 out of 15 particles labeled with either 5 mole% or 20 mole% NBD-lipid.

The confocal analyses just described do not provide insight into the organization of lipid at the particle surfaces due to the limits of optical resolution. We thus investigated the surface structure of PLGA lipid-enveloped nanoparticles by Cryo-TEM. Samples were embedded in ice by blotting a particle suspension (3 μL) on a 1.2/1.3 μm holey carbon-coated copper grid (Electron Microscopy Sciences) and immediately freezing the sample in liquid ethane using a Leica plunge-freezing machine. Samples were transferred to a cryogenic holder and imaged using a JEOL 2200FS transmission electron microscope at 185 μA emission current and 40,000X magnification. To provide a benchmark for the observation of membrane structures on solid particles, we first reproduced the work of Mornet et al. in forming lipid bilayers on 100 nm silica nanoparticles (Polysciences, Warrington, Pennsylvania) ⁷. As reported, a distinct electron-dense band exterior to an electron-light band was observed around the core of silica particles (see Supporting Information). Mornet et al. interpreted this structure to reflect the formation of lipid bilayers around the particles, with the electron-dense band a signature of the outer leaflet phospholipid headgroups, the light band arising from the acyl chain interior of the bilayer, and the inner leaflet phospholipid headgroups obscured by tight apposition to the electron-dense silica core. Using a weight ratio of 25:1 PLGA:DMPC (Figure 3a and b) or equimolar PLGA:HIV membrane components (Figure 3c) for emulsion synthesis of lipid-enveloped PLGA, we again observed lipid ‘shells’ enclosing polymer nanoparticles. As with bilayer coatings on silica, lipid assembly at the nanoparticle surfaces was typically detected as an electron-dense band exterior to an electron-light band. A second inner electron-dense band indicating phospholipid headgroups of an inner bilayer leaflet apposed against the polymer

core was sometimes observed (arrows on magnified view of Figure 3b) but was not resolved on the majority of particles, as was the case with bilayer-coated silica. However, after incubation of these particles in pH 7.4 phosphate-buffered saline for one week at 25°C to allow partial hydrolysis of the polyester cores, partially-delaminated lipid bilayers were clearly observed at the particle surfaces, (Figure 3d and e). Notably, mixing ‘bare’ surfactant-free PLGA nanoparticles with DOPC liposomes, the same process used to coat silica nanoparticles with bilayers, led to no liposome-PLGA particle fusion (Figure 3f and Supporting Information), suggesting that the lipid layers observed in Figures 3a–e are unlikely to have arisen from the fusion of free liposomes with already-formed polymer particles. We conclude that the lipid coatings observed on PLGA nanoparticles result from segregation of the lipid from the nascent particle bulk to the surface during solvent evaporation, and for a 25:1 weight ratio of PLGA:lipid, phospholipids self-assemble as single bilayers at the surface of the nanoparticles during this process.

Lipid-enveloped particle syntheses using a polymer:DMPC weight ratio of 25:1 contain enough lipid to theoretically coat a monodisperse population of nanoparticles ~230 nm in diameter with a single bilayer. Increasing the lipid:polymer ratio could lead to the formation of multilamellar lipid coatings on particles, or alternatively, the shedding of excess lipid as free liposomes. To determine which of these alternatives occurs, we next explored how increasing the lipid:polymer weight ratio impacts the morphology of lipid-enveloped nanoparticles. Increasing the quantity of lipid 9-fold (25:9 wt:wt PLGA:lipid) led to dramatic changes in the nanostructures of the lipid coatings, which depended also on the lipid composition. Using only neutral, zwitterionic DOPC as the lipid coating, we observed multilamellar onion-like stacks of lipid surrounding the core polymer (Figure 4a and b). However, use of a mixture of neutral and negatively-charged phospholipids (4:1 mol:mol DOPC:DOPG) abrogated this multilamellar stacking behavior, leading to formation of particles with single bilayer lipid surface layers and free liposomes (see Supporting Information).

Addition of 1 or 10 mole% DOPC-PEG (2 kDa PEG chain attached to the DOPC headgroup) to DOPC as the lipid component of syntheses containing a 25:9 weight ratio of PLGA:lipid also abrogated formation of multilamellar stacks, but instead of excess lipid being shed as free liposomes, the presence of PEG-lipid led to the formation of a novel ‘flower’ nanostructure in which the lipid coating was extruded into lipid bilayer ‘petals’ extending from the polymer core (Figure 4c and d). When incorporated into a liposome, PEG chains of this molecular weight anchored to the bilayer assume a mushroom-like configuration at concentrations below 4 mole % of the total phospholipid content; above 4% the PEG chains impinge on one another and form a brush-like configuration^{40–42}. We observed ‘flower’ formation for DOPC lipid mixtures containing either 1 mole% or 10 mole% PEG-lipid, where the PEG chains are expected to be in either the mushroom or brush-like states, respectively. Lipid structures extending away from the surfaces of particles were also directly observed on PLGA microparticles formed with these compositions, suggesting that the structures are not an artifact of cryoEM sample preparation (data not shown). The addition of PEG-lipid lowers the free energy penalty for curvature of lipid membranes⁴³, stabilizing smaller-diameter liposomes⁴⁴. The role of PEG-lipid in formation of polymer/lipid ‘flowers’ may thus be twofold: first, disrupting the stacking behavior of lipid bilayers by protruding from the bilayer surface, and second, stabilizing the higher curvature of the ‘petals’ compared to conformal coatings around the particles. For either the ‘onion’ or ‘flower’ lipid-enveloped particle structures to be of practical interest, they should have reasonable degrees of stability. Importantly, nanoparticles with either of these morphologies could be lyophilized with 20 mg/mL sucrose as a cryoprotectant using a Labconco freeze-dryer, and later reconstituted in water, preserving their nanostructures (see Supporting Information). Whether polymer-supported ‘onions’ or unusual nanostructures such as ‘flowers’ may be useful as biological tools or for drug delivery remains to be determined; however, the striking influence of lipid composition on the structure of

polymer/lipid nanoparticle surfaces highlights the need for careful structural characterization in synthesizing particles via self-assembly processes, where not only the intrinsic chemistry but also nanoscale surface structure may be dramatically altered by changes in phospholipid composition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Human Frontier Science Program, the NSF (award 0348259), and DARPA. A.B. was supported by fellowships from the Fannie and John Hertz Foundation and the Paul and Daisy Soros Foundation. We gratefully acknowledge the technical assistance of Dr. Kazuyoshi Murata in Cryo-TEM.

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Figure 1. Synthesis of lipid-enveloped microparticles and nanoparticles

(a) Schematic illustrating emulsion synthesis. (b, c) Scanning electron micrographs of lipid-enveloped microparticles (b) and nanoparticles (c) recovered by centrifugal separation.

Figure 2. Confocal laser scanning micrographs of lipid-enveloped microparticles

(a) Particles formed in deionized water with ~25:1 wt:wt PLGA:DMPC, labeled by inclusion of 1 mole% DMPC-rhodamine (left panel) and 1 mole% DSPC-PEG-biotin (detected by staining the particles with Alexa Fluor 488-conjugated streptavidin, middle panel) showed enrichment of lipids at the particle surfaces (right panel: brightfield image). Linescans of the fluorescence intensities through the centers of individual particles (lower panel) suggest that lipid is also present at lower levels in the bulk of the particles. (b) Particles formed with ~25:1 wt:wt PLGA:DOPC using PBS as the external aqueous phase showed DOPC-rhodamine fluorescence throughout the polymer core. (c, d) HIV-mimic particles made with PLGA and a lipid/sphingomyelin/cholesterol mixture mimicking the membrane composition of HIV-1 virus. Rhodamine-DOPC tracer in these particles was surface-enriched (c), but cholesterol-NBD tracer showed cholesterol throughout the polymer core (d). (e) Fluorescence recovery after photobleaching of a DOPC lipid-coated microparticle labeled with NBD-DOPE (20 mole % of lipid). A wedge-shaped area was photobleached, and total fluorescence intensity was then monitored from the bleached area (red trace), the entire particle (green trace) and a nearby background region (blue trace). Scale bars: 2 μ m.

Figure 3. Cryo-TEM micrographs of lipid-coated particles

(a,b) Particles synthesized with a 1:25 weight ratio of DMPC to PLGA were enveloped by single shells of lipid resembling previously published Cryo-TEM micrographs of lipid-coated silica nanoparticles. (c) Similar lipid structures were resolved at the surface of PLGA particles prepared with a lipid membrane composition mimicking the envelope of HIV-1 virus. (d, e) After a 1-week incubation in PBS, lipid could be seen delaminating and bulging from particle surfaces. (f) Images of surfactant-free PLGA particles incubated with small unilamellar vesicles did not show evidence for rupture or adsorption of liposomes onto bare PLGA particle surfaces. Boxed insets of (a, b, f) are shown magnified in right panels; arrows in (b, d) highlight bilayer structures. Scale bars: black bars, 100 nm; white bars, 50 nm.

Figure 4. Figure 4. Cryo-TEM micrographs of lipid-enveloped particles made with ~25:9 wt:wt PLGA:lipid

(a, b) PLGA:DOPC particles exhibit 'onion' morphologies, with multilamellar stacks of lipid packed together in conformal rings around the particle core. Boxed insets are shown magnified in the right panel of each image. (c, d) When 10 mole% PEG-conjugated lipid is included with DOPC as the lipid component, lipid 'flowers' form, with 'petals' extruding from the polymer core. Scale bars: 100 nm.