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Liposomes with Double-stranded DNA Anchoring the Bilayer to a Hydrogel Core

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Abstract

Liposomes are important biomolecular nanostructures for handling membrane-associated molecules in the lab and delivering drugs in the clinic. In addition to their biomedical applications, they have been widely used as model cell membranes in biophysical studies. Here we present a liposome-based model membrane that mimics the attachment of membrane-resident molecules to the cytoskeleton. To facilitate this attachment, we have developed a lipid-based hybrid nanostructure in which the liposome bilayer membrane is covalently anchored to a biocompatible poly(ethylene) glycol (PEG) hydrogel core using short double-stranded DNA (dsDNA) linkers. The dsDNA linkers connect cholesterol groups that reside in the bilayer to vinyl groups that are incorporated in the cross-linked hydrogel backbone. Size exclusion chromatography (SEC) of intact and surfactant-treated nanoparticles confirms the formation of anchored hydrogel structures. Transmission electron microscopy (TEM) shows ~100 nm nanoparticles even after removal of unanchored phospholipids. The location of dsDNA groups at the hydrogel-bilayer interface is confirmed with a fluorescence assay. Using DNA as a linker between the bilayer and a hydrogel core allows for temperature-dependent release of the anchoring interaction, produces polymer nanogels with addressible hybridization sites on their surface, and provides a prototype structure for potential future oligonucleotide drug delivery applications.

Keywords

Hydrogel-anchored liposomes; PEG nanogel; dsDNA anchors; stability; size exclusion chromatography

Liposomes are well-studied nanostructures with a variety of biomedical applications, useful for drug encapsulation and molecular targeting.^{1, 2} In addition to their biomedical applications, liposomes have long been used as model cell membranes to study such biophysical properties and phenomena as membrane permeability,³ mechanical stress relaxation,⁴ and fusion.⁵ In real cell plasma membranes, covalent anchoring between the bilayer and the underlying polymeric cytoskeleton plays an essential role in biophysical processes, radically affecting lipid diffusion,⁶ phase structure,^{7, 8} and mechanics.⁹ Constructing model liposome membranes that can recapitulate the effects of cytoskeletal anchoring requires forming both a biomimetic “cytoskeleton” in the core of the liposome and a mode of anchoring between the membrane and this cytoskeleton.

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Supporting Information Complete list of materials, experimental details, and supporting figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

There have been several efforts to construct liposomes with hydrogel cores that mimic the cytoskeleton. In early work, Torchilin et al. reported a free radical polymerization method for making acrylamide gel in ~600 nm vesicles.¹⁰ In 1995, Monshipouri and Rudolph cross-linked alginate in large liposomes.¹¹ Anchoring of the bilayer to an underlying hydrogel in liposomes was reported by Stauch and coworkers in 2002: they used a membrane-inserted anchor monomer with a polymerizable head group.¹² Several other approaches have been proposed for anchoring a liposome's lipid bilayer to an interior hydrogel.¹³⁻¹⁵ Phospholipid bilayers have also been self-assembled on preformed lipid anchor-containing hydrogel beads¹⁶ and fabricated on hydrophobically modified core-shell hydrogel spheres.¹⁷ Immobilization of liposomes onto hydrogel microbeads via avidin-biotin binding has also been previously reported.¹⁸

Anchoring the membrane to a hydrogel can greatly improve membrane stability. This is seen, for instance, in the radical increase of stability of hydrogel-anchored planar bilayers.¹⁹ Other efforts to improve liposome stability have focused on introducing synthetic components that allow for bilayer polymerization or provide a steric buffer between the bilayer and its environment.^{13, 20, 21} Generally methods for increasing liposome stability include the use of photopolymerizable phospholipids,^{20, 22, 23} making a polymer scaffold in the lipid bilayer,²⁴ coating the liposome surface with various polymers or nanoparticles,²⁵⁻²⁹ encapsulating polymer in the interior of liposomes,^{30, 31} and fabrication of hybrid phospholipid-block copolymer structures.³²⁻³⁴

Here we present a liposome/nanogel structure in which anchoring of the bilayer to the hydrogel core is accomplished by a double-stranded DNA linkage. These structures present remarkably stable intact bilayers, and they can be used as biomimetic model membranes that controllably recapitulate the connectivity between the cytoskeleton and the plasma membrane. The connectivity is thermally reversible, as the bilayer-anchoring cholesterol molecule is released from the cytoskeleton above the DNA melting temperature. By using the bilayer as an organizing architectural principle, we are able to construct nanogels in which the location of the oligonucleotide is well defined. After stripping the lipid from the nanogels, we are left with polymer nanostructures decorated specifically on their surfaces with oligonucleotides. These are potential sites for further molecular assembly and can be used to facilitate directed assembly of nanogels, allowing for mesoscale networks of networked nanoparticles.³⁵ These structures also represent potential prototypes for oligonucleotide drug delivery vehicles, building on significant previous work that has gone into designing polymeric and liposomal particulate drug delivery systems.^{15, 36, 37}

DNA-directed organization and manipulation of liposomes has recently been implemented as a tool for controlling interactions between nanoscale lipid bilayer structures.³⁸⁻⁴⁰ DNA molecules can act also as controllable and programmable building blocks for the accurate spatial arrangement of biomolecules such as proteins.⁴¹ DNA-lipid conjugates, such as cholesterol-tagged DNA, have been recently used for liposome tethering and studies of interactions between individual lipid vesicles.⁴²⁻⁴⁴ In this study, cholesterol-conjugated double-stranded DNA (dsDNA) is incorporated into the bilayer. The complement of the cholesterol-conjugated strand is terminated with a vinyl group that is incorporated in the hydrogel during free radical polymerization of PEG-diacrylate (PEG-DA) macromonomers (Figure 1).

The dsDNA anchor molecule was composed of two complementary single-stranded DNA (ssDNA) molecules: Cholesterol-tetra(ethylene glycol)-modified ssDNA (chol-DNA-1) and acrydite-modified ssDNA with a sequence complimentary to that of DNA-1 (acry-DNA-2) (each 15 bases long, sequences in supporting information (SI)). To hybridize, ssDNAs were dissolved in a 1:2 (v:v) mixture of acetonitrile and phosphate buffered saline (PBS, 150 mM

NaCl, 10 mM phosphate, pH 7.5). Oligonucleotides were annealed by incubating an equimolar mixture at 90°C for 3-5 min and gradually cooling to room temperature prior to addition to the lipid solution. Hydrogel-anchored liposomes were prepared by dissolving cholesterol-conjugated dsDNA and dioleoylphosphatidylcholine (DOPC) in a chloroform/methanol solution at molar ratio of 1:125. The mixture of DOPC/dsDNA anchor was dried and the resulting lipid film was rehydrated in a hydrogel precursor solution containing poly(ethylene) glycol diacrylate (PEG-DA, 10% (v)) and poly(ethylene) glycol methacrylate (PEG-MA, 5% (v)) along with ammonium persulfate (APS) initiator (0.2% (w/v)) and TEMED (0.2% (v)) in PBS. Rhodamine-methacrylate (rh-MA) was included in the monomer mixture at 24 μ M to provide a covalently incorporated fluorescent tracker. The dried lipid film was rehydrated by sonicating (10 s) at reduced temperature (4 °C) to prevent polymerization.

Following lipid rehydration, the liposomes were extruded at 4 °C through a polycarbonate filter to produce a monodisperse sample. The liposome suspension was diluted to prevent polymerization of PEG-DA and PEG-MA macromonomers outside of the liposomes. Since these monomers are relatively large and hydrophilic, they are trapped in the liposome interior. Their concentration inside the liposomes remains constant while they are diluted to a non-polymerizing concentration outside of the liposomes. The liposome suspension was brought to 40 °C to trigger polymerization of the acrylate and methacrylate groups inside the liposomes. This thermally controlled method for forming hydrogels in liposomes has not been previously reported; in fact, we know of only two previous reports of PEG-based hydrogels in liposome cores.^{45, 46} The vinyl group of the cholesterol-dsDNA anchor was incorporated into the hydrogel during polymerization.

The nanoparticles were characterized using size exclusion chromatography (SEC), transmission electron microscopy (TEM), and dynamic light scattering (DLS). Figure 2A shows chromatograms of liposomes formed by the temperature-controlled technique described above, but without the cholesterol-dsDNA anchors. These chromatograms track the fluorescence of the rh-MA included in the liposome core. SEC was performed with a Sephacryl 200 gel that allowed liposome-sized structures to elute in the void volume while retaining smaller species. As can be seen in Figure 2A, intact hydrogel-liposomes, liposomes without hydrogel, and hydrogel-liposomes treated with a high concentration of CHAPS surfactant have almost the same retention time in the column (and therefore a hydrodynamic radius (R_H) larger than the fractionation range of the column). On the other hand, the fluorescence from a surfactant-treated liposome sample prepared without monomer or initiator has a much longer retention time due to release of rh-MA from surfactant-lysed liposomes. These results indicate that hydrogels are formed by the process described above and that even after stripping the lipid bilayer with surfactant these hydrogels migrate as large particles, like the intact liposomes. TEM images also show the presence of nanoscale hydrogels (~80 nm) associated both with intact hydrogel liposomes and surfactant-treated nanogels. These images of partially dried hydrogels do not show the associated lipid bilayer; rather, they establish the successful fabrication of hydrogel cores (Figure 2B). The size of these nanoparticles is in good agreement with pore size of polycarbonate filter used for extrusion of liposomes. Association of the lipid bilayer with hydrogel in samples without surfactant treatment was confirmed by tracking rhodamine-labeled lipids (rh-DPPE) included in the lipid bilayer (see SI).

The insertion of the dsDNA anchor molecule in the lipid bilayer and conjugation of the anchor to the hydrogel were confirmed by SEC of liposomes in which the dsDNA anchor molecule had been dyed with a dsDNA-sensitive dye, Hoechst 33342 (H33342). H33342 complexes with the minor groove of dsDNA, resulting in a significant increase of the dye's quantum yield.⁴⁷ As shown in Figure 2C, intact anchored hydrogel-liposomes and

surfactant-treated anchored hydrogel-liposomes in which unanchored lipids were stripped off had the same retention time in SEC column, indicating that the dsDNA is associated with the hydrogel. Control experiments with surfactant-treated dsDNA-liposomes show the fluorescent species with a much higher retention time, corresponding to the surfactant-solubilized DNA anchor molecule with a size much smaller than that of the intact liposomes.

The size of the dsDNA-conjugated nanogels that had been treated with surfactant to strip the bilayer was compared to that of the intact dsDNA-conjugated hydrogel liposomes by DLS. The measured R_H of surfactant-stripped dsDNA-conjugated nanogels (53 ± 31 nm) was slightly smaller than that of the intact dsDNA-anchored hydrogel-liposomes (64 ± 10 nm), but the size distributions overlapped substantially. No significant change in size of anchored hydrogel-liposomes was observed after 5 weeks. We exposed surfactant-stripped hydrogels to organic solvent to assure that the hydrogel remained intact. Following treatment with chloroform, the size of the particles remained essentially unchanged (see SI). We also exposed the intact liposomes to DNase to evaluate whether there were any surface-accessible dsDNA molecules present. Our results (see SI) indicate that no H33342-associated low-molecular-weight species is generated upon DNase treatment, suggesting that all dsDNA molecules are located on the interior of the liposomes. This is likely due to the strongly polar nature of the DNA moiety leading to partitioning of surface-accessible dsDNA anchor molecules into bulk solution.

We used H33342 fluorescence to probe the position and accessibility of dsDNA species on nanogels. The quantum yield of H33342 increases about 10 times when it complexes with dsDNA.⁴⁸ We prepared surfactant-stripped nanogels from dsDNA-anchored hydrogel liposomes. SEC was used to separate these nanogels from the lipid/surfactant mixture that results from the surfactant treatment. SEC was performed either at room temperature or above the melting temperature (T_m) of the DNA-1/DNA-2 duplex (65°C , compared to a theoretical melting temperature of 55°C). SEC above the T_m leads to DNA melting, separation of the chol-DNA-1 species from the nanogels, and a nanogel product containing only single-stranded acry-DNA-2. We refer to this product as an ssDNA-nanogel while the product containing the hybridized duplex (prepared by SEC at room temperature following surfactant stripping) is referred to as a dsDNA-nanogel.

To confirm that the DNA anchor molecules are at the surface of the nanogels and solvent-accessible, we pre-incubated both nanogel species with 0.02 nM H33342 and then added increasing concentrations of DNA-1. As shown in Figure 3, there was a significant increase in H33342 fluorescence intensity (FI) upon addition of DNA-1 to the ssDNA-nanogel species. This was accompanied by a blueshift of the H33342 emission maximum wavelength (λ_{max}), as is known to occur upon the dye's binding to dsDNA.⁴⁸ Above a concentration of ~ 9 NM DNA-1, no further changes in FI or λ_{max} were observed, indicating that all available single-stranded acry-DNA-2 was hybridized. Similar shifts were not observed with the addition of DNA-1 to the ds-DNA nanogel because the H33342 in these experiments was bound to the dsDNA on the nanogel species; further hybridization could not be observed. These results indicate that the DNA anchor molecules are localized to the nanogel-bilayer interface and that when the bilayer is stripped off of the liposomes, DNA anchors are accessible and available for further assembly processes via melting and hybridization. Based on the saturation concentration of the complementary oligonucleotide strand, the measured absorbance of nanogel-associated rh-MA, and the DLS-determined average nanogel diameter, we estimate that ~ 20 DNA molecules are solvent-accessible on the surface of each nanogel particle (see SI).

The stability of hydrogel-anchored liposomes was examined with a fluorescence quenching experiment. A membrane impermeable dye (ANTS) and fluorescent quencher

(DPX) were co-encapsulated in liposomes prepared as described above. This dye-quencher combination has been utilized widely for liposome leakage studies;²⁶ ANTS quantum yield increases as it leaks from liposomes and the average dye-quencher distance increases. Liposomes were incubated in 20% fetal bovine serum at 37°C. Figure 4 shows that hydrogel-anchored liposomes were stable up to 136 h in serum, with ~22% release observed over 184 h. In contrast, unanchored liposomes showed rapid release of dye upon incubation in serum, with 100% release achieved after 184 h. Liposomes containing hydrogels but without anchors showed significant release after ~90 h and complete release after 184 h. These observations demonstrate the role of anchoring in the successful formation of stable liposomes as a model for biological membranes studies. In a complementary experiment, DLS showed a significant increase in the size of plain liposomes and hydrogel-liposomes incubated in 20% serum for 130 h (SI, Figure S3). No significant size change was observed for hydrogel-anchored liposomes. Covalent anchoring likely increases the energy necessary to delaminate the bilayer from the hydrogel surface, making it more difficult to form aggregated structures. Dye release data (Figure 4) and the time course of vesicle size as observed by DLS (see SI) indicate that membrane instability leading to dye release is accompanied by vesicle aggregation.

To demonstrate that the presence of the hydrogel did not impact the ability to package a payload in the liposomes, we encapsulated a fluorescently labeled peptide (sequence GTRQVTQASSFTWRVPG) (0.1 mg/mL) in core of liposomes both with and without hydrogel precursors during the liposome hydration process. Free peptides were removed by overnight dialysis in PBS and passing the liposomes over a Zeba spin column (40kDa MWC). The concentration of encapsulated peptides was calculated by measuring the absorbance of the dye in the same volume of liposomes. No significant difference in encapsulation efficiency could be observed (10% and 8% encapsulation for hydrogel-liposomes and plain liposomes, respectively). The hydrogel precursor solution is only 15 wt % monomer, and all monomers have molecular weights less than 600 Da. This solution would not be expected to exclude a relatively low-molecular-weight peptide cargo from the vesicle interior during liposome formation.

In conclusion, we have fabricated stable nanogel liposomes in which dsDNA anchors the bilayer to the underlying hydrogel. SEC and TEM demonstrated the formation of hydrogels, and a dsDNA-binding dye confirmed the location of the DNA anchors at the surface of the hydrogel. In this work, we have demonstrated temperature control of the polymerization process to construct complex supermolecular polymer-based structures without the use of photoinitiators. These structures incorporate a unique acrylate-DNA-cholesterol macroamphiphile that both participates in lipid bilayer self assembly and serves to stabilize lipid bilayers once formed. This allows for precise localization of the DNA molecules within the nanostructure. This DNA-programmable ~100 nm, highly stable liposome structure, mimicking cytoskeletal connectivity to the plasma membrane, has potential applications in biophysical studies of biomembranes. DNA melting can be used to remove the anchoring, allowing for the effects of anchoring to be specifically studied. These structures are potential prototypes for drug delivery vehicles. In a drug delivery application, the eventual fate of the hydrogel core would be important to the performance of the particles. This fate could be controlled by modifying the core to be a degradable PEG hydrogel, subject to hydrolysis or degradation of cross-linking disulfides in the cytosolic environment.⁴⁹ With the bilayers stripped off, the nanoparticles produced here have well-defined surface-localized DNA hybridization sites that can be used for the directed assembly of additional DNA-conjugated molecules or the assembly of the nanogels themselves into mesoscale structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic representation of a lipid bilayer anchored to a PEG hydrogel by a DNA linker. Cholesterol-conjugated dsDNA is covalently anchored to the hydrogel during free radical polymerization.

Figure 2.

(A) Normalized SEC chromatograms tracking rh-MA in different liposome species. Intact and surfactant-treated hydrogel liposomes elute in the void volume, showing shorter retention time than surfactant-treated liposomes with no hydrogel, indicating that a surfactant-resistant hydrogel is formed. (B) TEM images of negatively-stained hydrogel-

liposomes (left) and bare hydrogels formed by stripping away the bilayer with surfactant (right). Scale bars are 50 nm. (C) Normalized chromatograms tracking H33342 in various liposome species with dsDNA linkers. The retention time is the same for dsDNA anchor molecules in intact liposomes and in surfactant-stripped hydrogels.

Figure 3.

Changes in fluorescence intensity (ΔFI , red symbols) and maximum emission wavelength ($\Delta \lambda$, blue symbols) of 0.02 nM H33342 dye in the presence of ssDNA-nanogels (triangle) and dsDNA-nanogels (circle) as a complimentary DNA species (DNA-1) is added to the system.

Figure 4.

Dye release profiles from hydrogel-anchored liposomes, hydrogel liposomes without anchors, and liposomes without hydrogel (plain liposomes) incubated in 20% serum. Release profiles shows that hydrogel-anchored liposomes are significantly more stable than other types. The 100% point for each experiment is based on fluorescence intensity after total liposome lysis with surfactant. Each release experiment was performed three times; each data point shows the average over these three trials with error bars showing $\pm$ one standard deviation.