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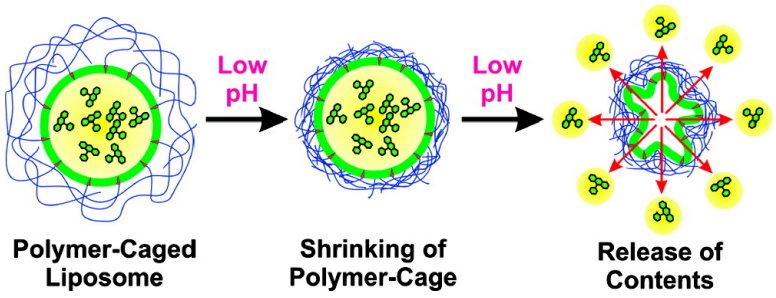

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Polymer-Caged Lipsomes: A pH-Responsive Delivery System with High Stability

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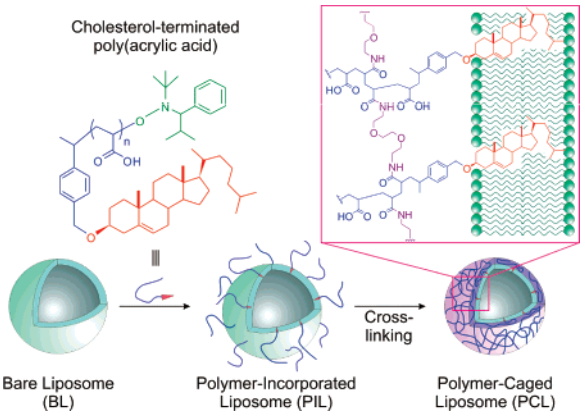
Liposomes are self-assembled vesicles consisting of a spherical bilayer structure surrounding an aqueous core domain. Due to their intrinsic biocompatibility and ease of preparation, several liposomal drugs have been approved.[1] In addition, modified liposomes on the nanoscale (~120 nm) have been shown to have excellent pharmacokinetic profiles[2] for the delivery of nucleic acids, proteins, and chemotherapeutic agents such as doxorubicin. However, major drawbacks of liposome-based drug carriers include their instability and the lack of tunable triggers for drug release. As such, there have been several attempts at enhancing the properties of liposomes.[1,3] Incorporation of polymerizable lipid amphiphiles leads to cross-linked liposomes with higher stability.[4] Unfortunately, every lipid system would require a specific polymerizable amphiphile, making this approach synthetically cumbersome. In addition, the cross-links are often too stable to allow for controllable releases of the payload. To provide a combination of stability and modification generality, hydrophilic polymers such as poly(ethylene glycol) (PEG)[2] and poly(*N*-isopropylacrylamide)[5] have been added to liposomes. However, these modifiers can easily dissociate from the liposome surface, returning them to the unstable state.[6] Herein, we demonstrate a general *drop-in* strategy that allows for long-term stabilization of virtually any liposome system via a biocompatible polymer cage.

Cross-linking of surface functional groups in self-assembled polymer nanoparticles has been shown to prevent the dissociation of polymer components.[7] In addition, highly functionalized polymer nanomaterials can be engineered to change shape via external stimuli such as pH and temperature.[8] Our work combines both of these design features to arrive at a single polymer amphiphile that can be used to stabilize any liposome system while allowing for additional attributes such as tunable drug-releasing properties and targeting ability. In particular, we demonstrate that a cholesterol-terminated poly(acrylic acid) (Chol-PAA) can be readily inserted into a known liposome system and then cross-linked to stabilize the bilayer membrane (Scheme 1). The resulting polymer-caged liposomes (PCLs) are highly stable—they can be lyophilyzed into powder forms and redispersed without loss of structural coherence. They can also be induced to release a model payload (calcein) under acidic conditions. As such, they may have applications as environment-specific nanoscale delivery vehicles.

Narrowly dispersed cholesterol-terminated poly(acrylic acid) (M_n = 2.5 kDa, M_w/M_n = 1.1) was synthesized via nitroxide-mediated controlled radical polymerization[9] of *tert*-butyl acrylate followed by acidolysis (Figures S1−S3 in Supporting Information (SI)). We employed poly(acrylic acid) as a hydrophilic polymer due to its biocompatibility and easily cross-linkable carboxylate group. The cholesterol end group acted as a single anchor to eliminate the possible aggregation often seen with polymers including multi-anchor groups.[10] Chol-PAA (10 mol % compared to the total amount of lipids) was mixed with a solution of bare liposomes (BLs, D_H = 82 ± 14 nm, PDI = 0.046 ± 0.021 via dynamic light scattering (DLS), prepared from dipalmitoyl-phosphatidylcholine, dioleoylphosphatidylglycerol, and cholesterol with molar ratio of 51.4:3.6:45, Figure 1A) and incubated overnight to yield polymer-incorporated liposomes[11] (PILs, Figure 1B). After the incubation, only particles with increased mean D_H (93 ± 15 nm, PDI = 0.047 ± 0.016) was observed, suggesting the homogeneous formation of PILs (Figure S4). Cross-linking of the poly(acrylic acid) moieties on the surface of PILs was achieved using 2,2′-(ethylenedioxy)-bis(ethylamine), and the formation of amide bond in the resulting PCLs (Figure 1C) was observed by water-suppressed ^{1}H NMR spectra (Figure S7). The apparent hydrodynamic diameter of the polymer-shell increased (Figure S9), indicating that significant cross-linking has occurred.

Scheme 1. Synthesis of Polymer-Caged Liposomes (PCLs)



Our PCLs are remarkably stable—their spherical structures were fully preserved after freeze-drying and rehydration (Figure 2A). In contrast, the same treatment completely destroyed the spherical BLs (Figure S6), presumably due to loss of the aqueous core as well as increased surface−surface interactions that allow for establishment of broad lamellae structures.[12] The outstanding stability of our PCLs under the lyophilization/rehydration process suggests that they can be stored on a long-term basis, a desirable feature in delivery applications.

For in vivo applications, drug carrier vesicles must possess high plasma stability. Accordingly, we evaluate the stability of a calcein-encapsulated sample of our PCLs against fetal bovine serum (FBS) at 37 °C following a procedure described by Allen et al.[13] As calcein's fluorescence is self-quenched under the high concentrations found in intact liposomes, its leakage due to vesicle rupture is readily observed. Remarkably, only a minute leakage of calcein (~5%) was observed from our PCLs after 500 h, an order of magnitude less than the leakage in BLs (Figure 2B). We attributed the inhibition of PCL rupture to the steric barrier provided by the cross-linked polymer shell. Although incorporation of PEG-conjugated phospholipids into liposomes has been reported to sterically stabilize the resulting vesicles,[2] long-term stability was still low due to the rapid dissociation of the water-soluble polymer-attached lipids from the bilayer membrane.[6b] In a similar manner,

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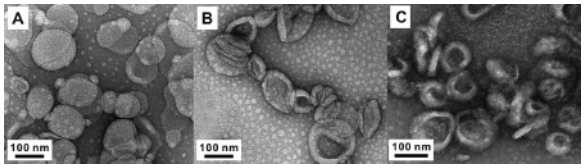

Figure 1. Transmission electron microscope (TEM) images of (A) bare liposomes (BLs), (B) polymer-incorporated liposomes (PILs), and (C) polymer-caged liposomes (PCLs). All samples were negatively stained with 4% uranyl acetate. Both wholly spherical (A) and indented spherical morphologies (B and C) are commonly observed in liposomal TEM (see SI).

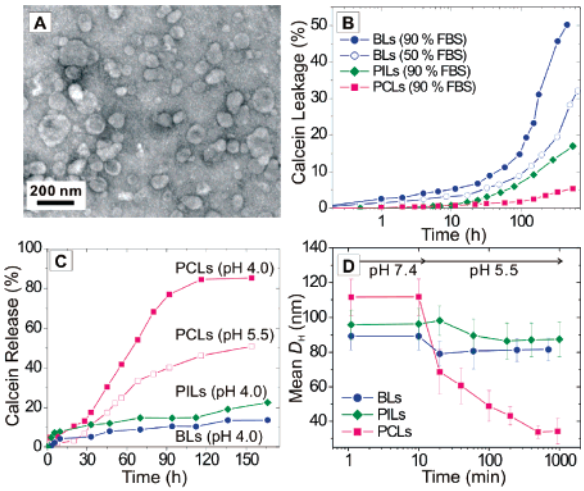

Figure 2. (A) TEM image of PCLs after freeze-drying and rehydration. (B) Calcein leakage assay of BLs, PILs, and PCLs at 37 °C in fetal bovine serum (FBS). (C) Acid-triggered calcein release at 37 °C and (D) temporal evolution of mean D_H in pH 7.4 and 5.5.

the noncross-linked Chol-PAAs in our PILs could dissociate easily into solution during prolonged incubation and lead to increased calcein leakage from the unprotected vesicles. This is indeed the case: calcein leakage from PILs was comparable to that of PCLs during the initial 10 h of incubation (~0.7% leakage); however, it began to increase steeply afterward. Similar lag periods (several minutes to a few hours) prior to accelerated destabilization were reported in PEG-conjugated liposomes.[6c] In this sense, the cross-linked polymer-shell in our PCLs greatly reduces polymer dissociation and results in a substantial increase in their long-term stability.

Given that a significant number of carboxylic acid groups remained on the cross-linked polymer-shell of our PCLs, we hypothesize that their shapes, and consequently their payload-release ability, may be manipulated with pH.[8] Hence, we were pleased to observe a pH-dependent release profile from our calcein-encapsulating PCLs: when they were subjected to acidic conditions at 37 °C, releases of 84% and 50% were achieved after 150 h at pH 4.0 and 5.5, respectively (Figure 2C). In comparison, relatively slow releases in BLs and PILs were observed at pH 4.0 over the same period. Evidence for the collapsed cross-linked shells can be found by monitoring the mean D_H of PCLs as the solution pH is suddenly reduced to 5.5 from 7.4 (Figure 2D): a rapid decrease of mean D_H commenced and continued to decrease over 500 min (69% reduction in diameter). As controls, BLs and PILs did not show significant decrease in mean D_H at pH 5.5. Low pH induces a random-coil-to-globular phase change for polymers in the PCL membrane due to increased hydrophobic interactions between polymer chains.[8] At the same time, the protonated acrylate groups in PCLs can hydrogen bond to the phosphodiester head groups of the lipid molecules in the membrane, decreasing lipid−lipid interactions responsible for membrane stabilization.[14] Both of these effects can lead to a collapse of the cross-linked shell and a subsequent compression/rupture of the PCL core, mechanically similar to that observed for a cross-linked poly(acrylamide-acrylic acid) hydrogel.[15] These effects perturb the membrane structure and may induce the formation of pores that are sufficiently large to allow for the leakage of the calcein contents.[16]

In summary, polymer-caged liposomes were readily prepared from preformed liposomes and a cholesterol-functionalized poly(acrylic acid) additive via a facile *drop-in* procedure. The highly enhanced stability and tunable pH-sensitive responses of these novel materials are made possible entirely by the environmental responsive properties of the encapsulating polymer shell. Our simple but highly effective strategy could be used to modify many clinically relevant liposome-based drug-delivery systems, including inorganic drug-encapsulated liposomes.[17] In addition, as the cross-linked polymer shell still possess unmodified carboxylic acid groups, it can be further functionalized with antibody- and ligand-based targeting groups using "post-particle-formation modification" strategies.[18] The results from such studies will be reported in due course.

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Supporting Information Available: Synthetic and preparative procedures and characterization data for Chol-PAA, BL, PIL, and PCL. This material is available free of charge via the Internet at http://pubs.acs.org.

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