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Thiol lysolipids undergo thiol-thioester exchange with two phenyl thioester-functionalized tails to produce phospholipid structures that assemble into liposomes with differences in exchange rates, temperature sensitivity, permeability, and continued exchange behavior. This *in situ* formation reaction imparts dynamic characteristics into the membrane for downstream liposome functionalization and mimics native membrane remodeling.

Synthetic liposomes, lipid structures[1] self-assembled with hydrophilic head groups extending into solution and hydrophobic chains sequestered into a bilayer core to minimize contact with water, effectively separate an aqueous compartment from the surrounding environment. With this assembly, liposomes mimic cell membrane structures and have garnered significant interest for their ability to encapsulate hydrophobic molecules within the bilayer core and hydrophilic components inside the internal compartment. These features have found applications in a wide range of fields from drug[2–9] and cosmetics[10–13] delivery to microreactors[14–19]. More recently, the pursuit of a bottom-up artificial cell[20–28] has garnered significant interest, frequently utilizing synthetic liposomes in place of the cell membrane.

Previous work examined the ability to generate synthetic phospholipids, and by extension liposomes, *in situ* using a few reactions[29–32]. For example, utilizing coupling reactions, such as the copper-catalyzed azide-alkyne cycloaddition reaction, an aliphatic tail is added to a phospholipid-based structure bearing a charged head-group and a single aliphatic tail (lysolipid). Coupling drives the conversion of micelles and stabilized oil droplets to liposomes, enabling *in situ* assembly of lipid bilayers which grow from the surface of the oil droplets[29]. However, little work has been done to incorporate reversible chemistries, resulting in systems that serve as poor mimics for a native cell membrane's enzymatic ability to exchange one tail for another[33]. What work has been done to integrate reversibility into the membrane has incorporated exchange reactions into surfactant tails[34,35] and bilayer-water interfaces[36,37] for membrane functionalization or utilized native chemical ligation and reversible native chemical ligation for phospholipid formation and exchange[38]. However, these systems were either not extended to phospholipids or required addition of more lysolipid precursors, limiting the number of exchanges possible before the bilayer becomes unstable due to disfavored packing.

Thiol-thioester exchange is a reversible reaction wherein a thiolate anion attacks the carbonyl carbon of a thioester resulting in a tetrahedral intermediate. Intermediates are able to break back down to the original compounds or to form a new thiol-thioester pair[39]. Exchange in this fashion continues as long as thiolate anions are present, resulting in a dynamic system capable of constant remodelling. Applying this behavior to an *in situ* formation method would enable exchange of the lysolipid and tail pairs to produce a liposome mimic of cell membrane remodelling. Additionally, with this behavior present in liposome systems it would be possible to incorporate new functionalities into a pre-formed bilayer by subsequent introduction of compounds bearing thioester groups. Herein, we report the use of this exchange reaction to develop a synthetic lipid bilayer capable of continued remodelling.

Dynamic behavior enabled by the thiol-thioester exchange was introduced to liposome membranes through design and synthesis of lysolipids bearing primary thiol functionalities (SHPC) [**1a**] and one of two aliphatic tails (C7 [**2**] and C11 [**3**]) terminated in phenyl thioesters as depicted in Scheme 1. Phenyl thioester functionalities were selected to drive the generation of synthetic phospholipid and free phenyl thiol due to the greater stability of the lipid thioester as compared to the phenyl thioester, as more basic thiols will exchange to produce more stable thioesters. This property drives tetrahedral

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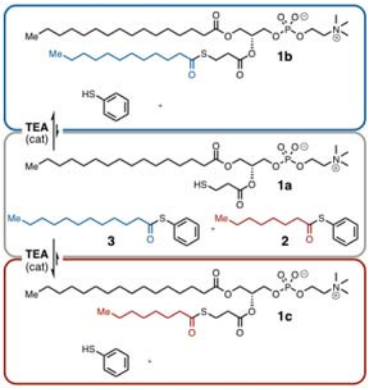
intermediates to break down into full phospholipids and phenyl thiols. Reaction progress was monitored on an analytical LC-MS equipped with an Evaporative Light Scattering Detector (ELSD) to track consumption of lysolipid and production of phospholipid product. SHPC (5mM), with residual TCEP, was mixed with either C7 or C11 thioester tails (5mM) in the presence of the base catalyst triethylamine (TEA) (10mM) to generate the reactive thiolate anions in water at room temperature. TEA, a weak base, was used sparingly to catalyze



Scheme 1. A schematic illustrating the formation of synthetic phospholipids using the thiol-thioester exchange reaction. Thiol-functionalized lysolipids [**1a**] undergo reversible exchange with either C7 [**2**] or C11 [**3**] phenyl thioesters in the presence of a basic catalyst, triethylamine (TEA), to generate phospholipid products [**1b** and **1c**]. These lipid products are then capable of assembling into bilayers to form liposomes.

the exchange without significantly increasing hydrolytic cleavage of the ester linkages. In the presence of C7 phenyl thioesters, SHPC was consumed over the course of 48 hours leading to approximately 90% conversion to a C7 thioester-containing phospholipid product (phospholipid **1c**) (Figure 1A,B). Exhibiting faster exchange, SHPC mixed with C11 phenyl thioester tails was consumed over 12 hours reaching nearly complete conversion to a C11 thioester-containing phospholipid product (phospholipid **1b**) (Figure 1C,D). In either reaction if the TEA catalyst was excluded, no formation of phospholipid product was observed over the same time frames (SI Figure S7), verifying the dependence on the catalyst presence.

Relatively slow reaction times in both cases are attributed to the necessity of a weak base catalyst as needed to prevent hydrolysis, the dilute reactants, and a stoichiometric ratio of thiol to thioester precursors. However, the ability of the reaction to progress despite these conditions conveys the strength the thiol-thioester exchange reaction offers the *in situ* formation system. Interestingly, C11 thioesters underwent more rapid exchange than the C7 thioesters, which may be due to a more favorable packing of the precursors to exclude water and thereby increase the lifetime of thiolate anion intermediates or to increase accessibility of thiolate anions to phenyl thioesters. Additional work would be necessary to determine the role tail hydrophobicity plays on the rate of the thiol-thioester exchange.

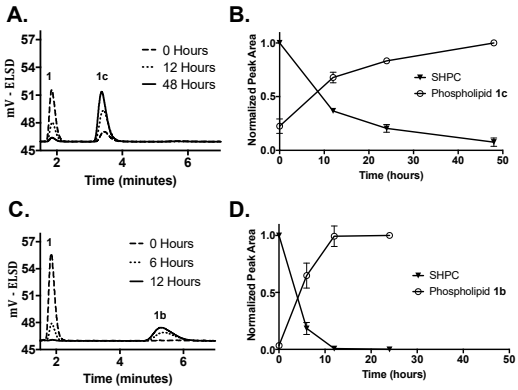


Figure 1. LC-MS-ELSD chromatography monitoring reactions of 5mM reduced thiol lysolipid (SHPC) [**1a**], 10mM TEA and 5mM thioester tail [**2** or **3**] (A, C). C7 thioester tails and SHPC, normalized to 0 hours, undergo approximately 90% conversion to phospholipid **1c,** normalized to 48 hours, over the course of 48 hours (B) and C11 thioester tails with SHPC, normalized to 0 hours, undergo nearly complete exchange over 12 hours to produce phospholipid **1b**, normalized to 24 hours (D).

Fluorescence microscopy and cryo-TEM were used to assess assembly of mixtures bearing the phospholipid products, as well as unreacted precursors and thiophenolate, into liposomes. Samples were prepared as in the LC-MS-ELSD experiments ([reduced SHPC]=[thioester tail]=5mM, [TEA]=10mM) with the addition of 2uM rhodamine-DHPE for fluorescence microscopy samples. Liposomes greater than a micron in diameter were observed under fluorescence microscopy following development of the C7 phenyl thioester-based reaction at room temperature for 48 hours (Figure 2A). Cryo-TEM images display single rings rather than stacked layers, signifying assembly into predominantly unilamellar structures (Figure 2B). These differences in imaged liposome sizes are attributed to reduction in shear forces when liposome solutions were developed in sealed glass slides for fluorescence imaging and the limit of detection utilizing fluorescence microscopy. Unilamellar assemblies are likely due to the slow reaction times and make this liposome formation method particularly useful for applications such as artificial cells and membrane dynamics studies in which these structures are desired for imaging[40].

Separately, it should be noted that phospholipid **1b** exhibited dependence upon temperature for the assembly of liposomal structures. If developed at slightly elevated temperatures such as 30°C, phospholipid **1b** assembled into liposomes several microns in diameter (Figure 2C); however, if the solution was developed at room temperature, no liposomes were evident. This temperature dependent assembly suggests that temperature plays a much stronger role in the packing of phospholipid **1b** than phospholipid **1c** near ambient conditions leading to these significant morphological transitions. In all cases, cryo-TEM displayed assembly into worm-like micelles (Figure 2D).

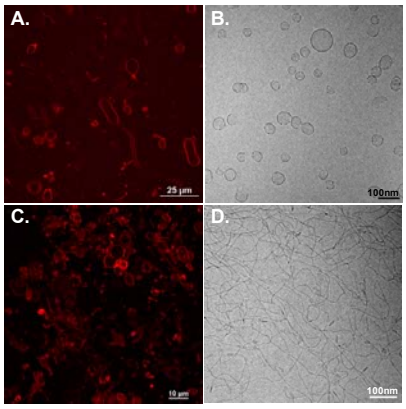


Figure 2. Fluorescence microscopy and cryo-TEM images of phospholipid assemblies. C7 thioester tails (5mM) undergo exchange with reduced SHPC (5mM) in the presence of 10mM TEA over 48 hours to produce predominantly unilamellar liposomes, as evidenced by fluorescence microscopy (A) and cryo-TEM (B). C11 thioester tails (5mM) undergo exchange with reduced SHPC (5mM) in the presence of 10mM TEA to produce liposomes at elevated temperatures, visualized using fluorescence microscopy following development at 30°C for 24 hours (C) and tubular micelles visualized using cryo-TEM following development at 40°C for 24 hours (D).

Under fluorescence microscopy, localization of rhodamine-DHPE to the membrane highlights the structures as closed membranes. However, to probe the integrity of the lipid bilayers as boundaries capable of encapsulating hydrophilic molecules, additional studies were necessary. Photo-bleaching of an encapsulated, charged dye followed by monitoring for recovery was used to examine the barrier to diffusion presented by the lipid membrane. Both vesicle populations displayed photobleaching of encapsulated HPTS immediately following irradiation, with phospholipid **1c** samples losing 74% of initial internal fluorescence intensity and phospholipid **1b** samples losing 45% of initial internal fluorescence intensity. In contrast, a control solution of 1mM HPTS resulted in a less than 4% decrease in fluorescence intensity following photobleaching, verifying that the phospholipid membranes remained intact following irradiation. For phospholipid **1c** samples, no increase in fluorescence within the membrane boundary was observed during the recovery period. Alternatively, restoration of fluorescence within phospholipid **1b** liposomes occurred over the course of 20 seconds after which it was not possible to consistently distinguish liposomes (Figure 3, SI video 1, 2), indicating that HPTS was able to diffuse from the external solution into the vesicle cavity. These results indicate that both phospholipids assemble into enclosed systems to enable the initial photobleaching, and indicate that phospholipid **1c** vesicles produce a more impermeable membrane at room temperature. The greater degree of permeability in phospholipid **1b** vesicles could be used for applications requiring a diffusion-limited system capable of taking up resources from the environment.

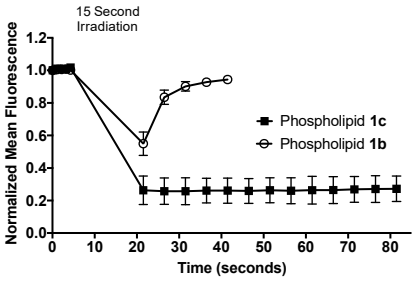


Figure 3. Photobleaching assays of phospholipid **1c** and phospholipid **1b** liposomes. Both systems enable bleaching of internal HPTS upon irradiation. Mean internal fluorescence normalized to initial fluorescence values shows restoration of fluorescence over 20 seconds for phospholipid **1b** systems and no restoration of fluorescence for phospholipid **1c** systems. Values were averaged for 3 and 6 liposomes for phospholipid **1b** and **1c**, respectively.

Due to the reversible nature of the thiol-thioester exchange reaction, it is possible to introduce new thioester-functionalized molecules following liposomal formation to incorporate new moieties into the lipid structures. This approach would enable downstream labelling or modification of the liposomes. To demonstrate this capability, following equilibration of the first thioester exchange reaction, an equimolar quantity of complementary aliphatic thioester tail was added. Samples were taken at various time points and injected onto the analytical LC-MS-ELSD to monitor exchange. Over the course of 96 hours, exchange of phospholipid **1b** with C7 phenyl thioester tails was apparent, demonstrating over 30% conversion of phospholipid **1b** to phospholipid **1c**, and fluorescence microscopy verified the persistence of liposomal structures (Figure 4). However, when the opposite tail and phospholipid exchange was attempted, there was no exchange of C11 phenyl thioester tails into the phospholipid **1c** system evident using either equimolar or excess quantities of C11 phenyl thioester. Together, these aliphatic tail exchange results promote the idea that phospholipid **1c** has a more stable thioester than phospholipid **1b** or that there are favorable lipid constructs that either prevent or facilitate exchange, respectively, in phospholipid **1c** and **1b**.

In this work a novel method for the *in situ* formation of synthetic phospholipids using a reversible reaction has been developed. Dynamic behavior in the bilayer was introduced using thiol-thioester exchange between SHPC and phenyl thioester tails. Two tails, C7 phenyl thioester and C11 phenyl thioester, exchange with SHPC to produce phospholipid products. These products assemble into liposomes capable of varying degrees of hydrophilic compound encapsulation. Beyond liposome assembly, phospholipid **1b** demonstrates the ability to undergo further exchange with C7 phenyl thioesters. This continued exchange can better mimic the remodelling present in native cell membranes which occurs daily[41,42] or seasonally[43,44], furthering the pursuit of a bottom-up artificial cell and enabling downstream modifications of liposomal

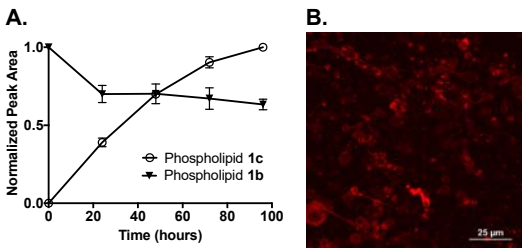


Figure 4. Phospholipid **1b** undergoes exchange with C7 phenyl thioester tails. Following formation of phospholipid **1b** over the course of 24 hours, equimolar C7 thioester tails [**3**] were added. Exchange of the two tails progressed over the course of 96 hours to convert over 30% of phospholipid **1b**, normalized to 0 hours, to phospholipid **1c**, normalized to 96 hours (A). Fluorescence microscopy verified persistence of liposomes following addition of C7 phenyl thioester tails after 48 hours of continued development at 30°C (B).

structures following *in situ* formation.

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Conflicts of interest

There are no conflicts to declare.

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