Fuel-Driven Phospholipid Vesicles with Temporal Control for Regulated Cargo Release

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Abstract: *Metabolism in biological systems involves the continuous formation and breakdown of chemical and structural components, driven by chemical energy. In specific, metabolic processes on cellular membranes result in in situ formation and degradation of the constituent phospholipid molecules, by consuming fuel, to dynamically regulate the properties. Synthetic analogs of such chemically fueled phospholipid vesicles have been challenging. Here we report a bio-inspired approach for the in situ formation of phospholipids, from water soluble precursors, and its fuel driven self-assembly into vesicles. We show that the kinetic competition between anabolic and catabolic-like reactions lead to the formation of double-tail phospholipid, which self-assembles into vesicles, and its eventual disintegration. Spectroscopic and microscopic analysis demonstrate the formation of transient vesicles whose lifetime can be easily tuned from minutes to hours. Importantly, our design results in the formation of uniform sized (65 nm) vesicles simply by mixing the precursors, thus avoiding the traditional complex methods. Finally, our sub-100 nm vesicles are of the right size for application in drug delivery. We have demonstrated that the release kinetics of the encapsulated cargo molecules can be dynamically regulated for potential applications in adaptive nanomedicine.*

Metabolic processes are essential functions of living systems, where chemical and supramolecular structures are cyclically synthesized (anabolism), by consuming chemical energy, and degraded (catabolism) using chemical reaction networks. Such processes enable the hallmark features of life such as adaptability and spatiotemporal control. Inspired by nature, synthetic supramolecular systems have been developed that continuously consume (chemical-) energy to sustain their structures. This energy input transforms simple precursor molecules into self-assembling components which are inherently unstable and revert to their original forms once the energy input (fuel) ceases, giving them a transient existence and thus temporally controlled. This has resulted in transient self-assembled nanomaterials[1]-[2] using various fuel[3] for application in enhanced catalysis, self-erasing inks, transient electronics, multiple helical states etc[2h, 3g, 4]. However, chemical fuel driven lipid vesicles are rarely investigated, and it would be of considerable interest for active material research and nanomedicine in particular.

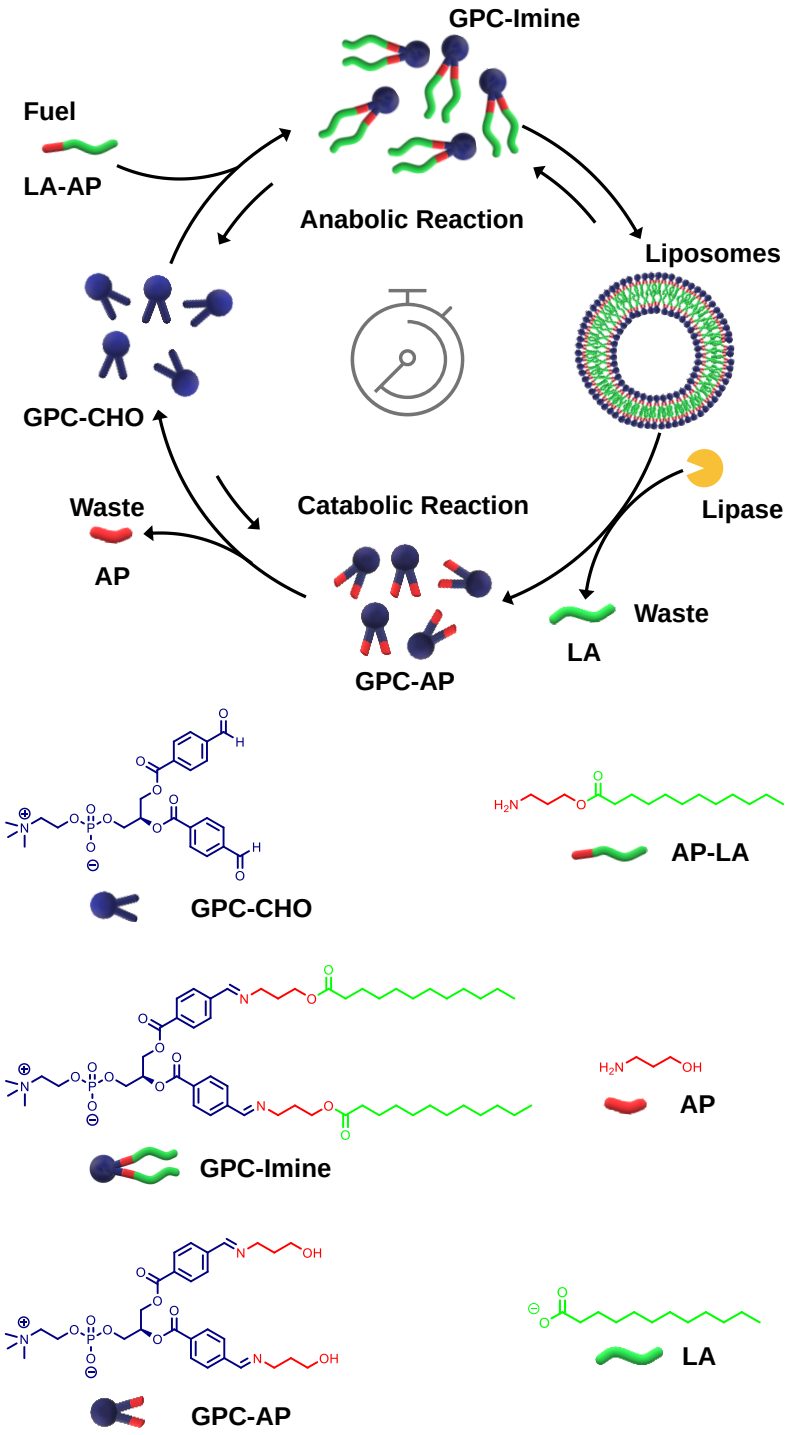
The self-assembly of phospholipids (PhL) into bilayers is the primary process in forming cell membranes. These bilayers are responsible for compartmentalizing chemical potential pools to sustain metabolism and ultimately support life. Synthetic analogs of PhLs have mostly utilized self-assembly of pre-formed PhLs as protocell models, delivery vehicles etc[5]. In contrast, PhLs are formed *in situ* in cells which provide the dynamic and adaptable nature to cellular membranes. In cells, an anabolic process consumes chemical energy like ATP to form PhLs, whereas the same PhLs are catabolically degraded by the membrane-bound enzymes like lipase[6]. Such metabolic processing of PhLs provides cells with dynamic, spatiotemporal characteristics. In this regard, Fletcher and co-workers have demonstrated the *in situ* formation of autocatalytic lipid micelles and vesicles[7], composed mostly of synthetic thioalkanes and not PhL. Devaraj and team

have reported *in situ* PhLs synthesis, which finally forms stable vesicles under equilibrium. However, they are not chemically fueled, thus are not cyclically formed and degraded and hence lack temporal regulation[8]. They have recently reported an example of fueled, temporally controlled PhL vesicles that demonstrate biological membrane plasticity[9]. However, it requires ester-forming coupling agents, and the catabolic hydrolysis is spontaneous and thus lacks temporal tunability. Hence, there is a need of novel design for the *in situ* formation of PhLs fueled by chemical energy, to form biomimetic lipid vesicles which can dynamically regulate cargo delivery.

We present an *in situ* synthesis of biomimetic PhL and their chemical fuel driven self-assembly under physiological conditions, resulting in vesicles with a programmable lifetime. The chemical design introduces an ester bond to form the PhL (anabolic reaction), which spontaneously self-assembles into a vesicle. Furthermore, the simultaneous presence of lipase results in the hydrolysis of the same ester (catabolic reaction) leading to disassembly (Scheme 1). However, *in situ* ester formation is challenging and usually requires coupling agents[10], which can have side reactions in the biological milieu. We have avoided it by using the design of amino-ester fuel[1e]. Here, the hydrophilic phosphorylcholine aldehyde (GPC-CHO) reacts with hydrophobic amino-ester fuel (AP-LA) to form an imine bond and thus results in a double tail PhL GPC-Imine containing ester unit (Scheme 1). Furthermore, like in a living cell, the presence of lipase hydrolyses the lipid GPC-Imine to disintegrate the vesicle and regenerate GPC-CHO, and a new cycle starts by utilizing fresh fuel. Additionally, we present a temporal control over the vesicle lifetime where the release kinetics of encapsulated cargo can be dynamically regulated. Thus, we present a novel design of *in situ* formation of PhL, from completely water-soluble precursors, and catabolic degradation of PhL, as a biomimetic approach to form chemically fueled transient vesicles.

The hydrophilic aldehyde derivative GPC-CHO and the aminopropanol ester derivative of lauric acid (AP-LA) as hydrophobic fuel (Scheme 1) was synthesized as described in Scheme S1-2. The chemical design of fuel was to ensure structural resemblance with biological lipids (Supplementary Calculation) and to retain water solubility. The amine of the fuel AP-LA reacts with the aldehyde of GPC-CHO to form amphiphilic GPC-Imine that self-assembles into vesicles. Additionally, the presence of lipase hydrolyses the ester bonds of the GPC-Imine, leading to the disassembly of vesicles along with waste formation, resulting in transient PhL vesicles (Scheme 1).

The kinetics of GPC-Imine formation (anabolic) was investigated by adding AP-LA to GPC-CHO at physiological conditions. The initial transparent reaction mixture became turbid within minutes after adding AP-LA, indicating the formation and self-assembly of GPC-Imine (Figure S1). The time-dependent high performance liquid chromatography-mass spectrometry (HPLC-MS) analysis confirmed the formation of double-tailed GPC-Imine with almost complete conversion within 20 minutes along with 5 percent of single-tailed intermediate (Figure 1a, Figure S2-S6). Furthermore, time-dependent 1H NMR showed the disappearance of the aldehyde signal at 10.02 ppm, indicating complete consumption of GPC-CHO within 20 minutes leading to the formation of self-assembled GPC-Imine (Figure 1b). Thus, NMR and HPLC analysis confirmed an almost complete conversion of GPC-CHO into GPC-Imine.



Scheme 1. Schematic representation of the *in situ* PhL formation and their transient vesicle assembly along with various chemical structures.

Since HPLC and NMR are time point measurements, we looked at the UV-Vis spectral changes to probe reaction in real time. The absorption spectra of GPC-CHO showed a maximum at 254 nm which upon conversion to GPC-Imine red shifted to 260 nm along with a shoulder at 282 nm (Figure 1c). Thus, a plot of time-dependent absorbance changes at 282 nm showed a gradual increase and plateauing of GPC-Imine concentration within 20 minutes, further confirming the HPLC and NMR analysis (Figure 1d, 1b).

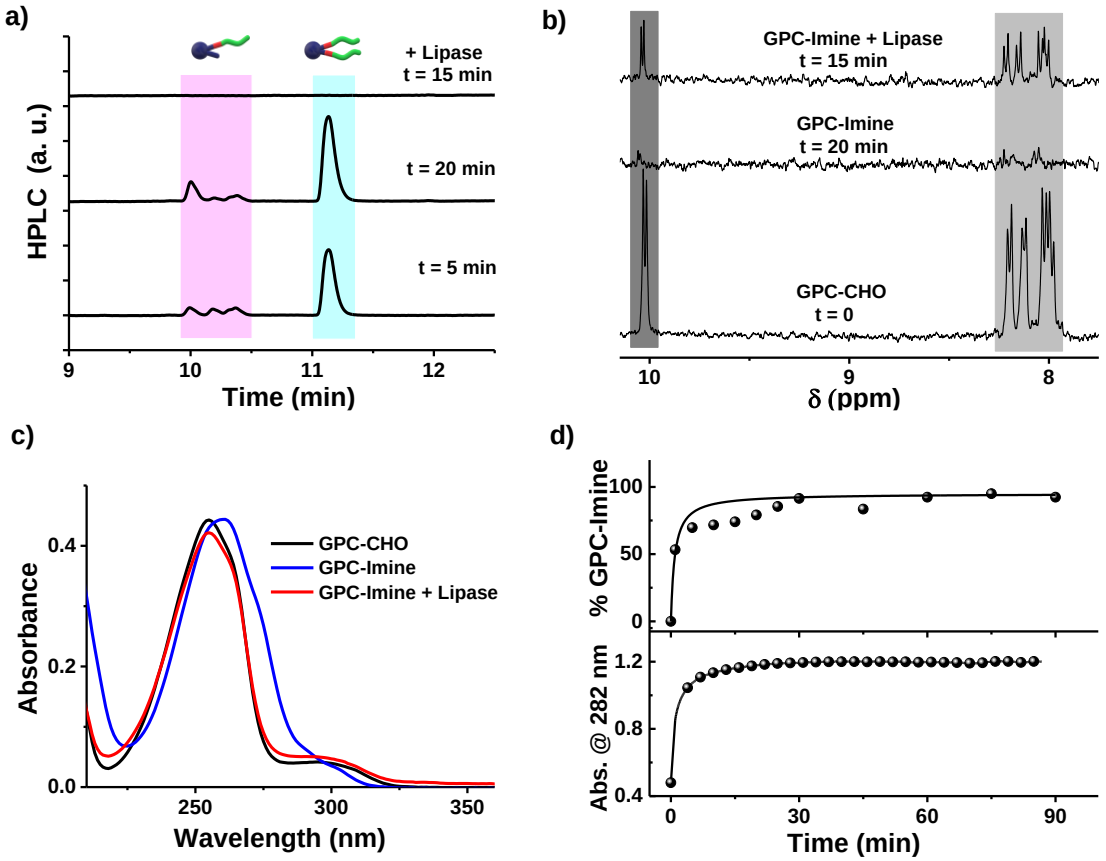


Figure 1. a) Time dependent HPLC analysis of GPC-Imine before and after lipase addition. Multiple peaks for single tailed lipid at ≈10.19 minutes correspond to chemically different possibilities (Figure S4); b) time dependent ^{1}H NMR showing the disappearance and reappearance of GPC-CHO (5 mM GPC-CHO, 15 mM AP-LA). c) Absorption spectra of GPC-CHO, GPC-Imine before and after hydrolysis with lipase, 0.1 mm cuvette. d) Kinetics of GPC-Imine formation obtained from HPLC peak area (top) and UV-Vis absorbance changes at λ= 282 nm (bottom, 1 mm cuvette). Conditions: 1 mM GPC-CHO, 3 mM AP-LA, excess lipase, pH 7.4 HEPES buffer.

Having described the formation kinetics, we probed the degradation (catabolic) reaction through lipase-catalyzed ester hydrolysis of GPC-Imine. The addition of an excess of lipase to preformed GPC-Imine resulted in the disappearance of the GPC- Imine and the reappearance of the GPC-CHO as confirmed by HPLC and NMR (Figure 1a-b). Absorption spectra after lipase addition were also very similar to GPC-CHO (Figure 1c), indicating ester hydrolysis of the GPC-Imine and regeneration of GPC-CHO. Interestingly, we did not observe the GPC-AP intermediate due to the spontaneous conversion of GPC-AP to GPC-CHO via imine hydrolysis (Scheme 1, Figure S7). Therefore, we have confirmed that lipase hydrolysis of the GPC-Imine regenerates GPC-CHO and produces waste.

To further probe the supramolecular transformation, dynamic light scattering (DLS) measurements were performed. The AP-LA solution showed small aggregates below 10 nm (Figure 2a, Figure S8). However, the *in-situ* formation of GPC-Imine resulted in around 65 nm nanostructure, with a very good correlation function, indicating uniform size (PDI=0.221). Furthermore, lipase-catalyzed degradation of GPC-Imine led to the loss of 65 nm DLS peak, indicating disassembly and the appearance of large aggregates (approx. 3.5μm). These large aggregates were confirmed to be the crystals of lauric acid (LA) waste generated during hydrolysis (Figure S9). The transmission electron microscopy (TEM)

micrographs of self-assembled GPC-Imine confirmed the formation of well-defined vesicles of around 60 nm in diameter. However, the AP-LA alone showed no observable morphology (Figure 2b-c, Figure S10). Moreover, after hydrolysis by lipase, large crystalline structures of waste LA were observed, indicating the complete disintegration of vesicles (Figure 2d).

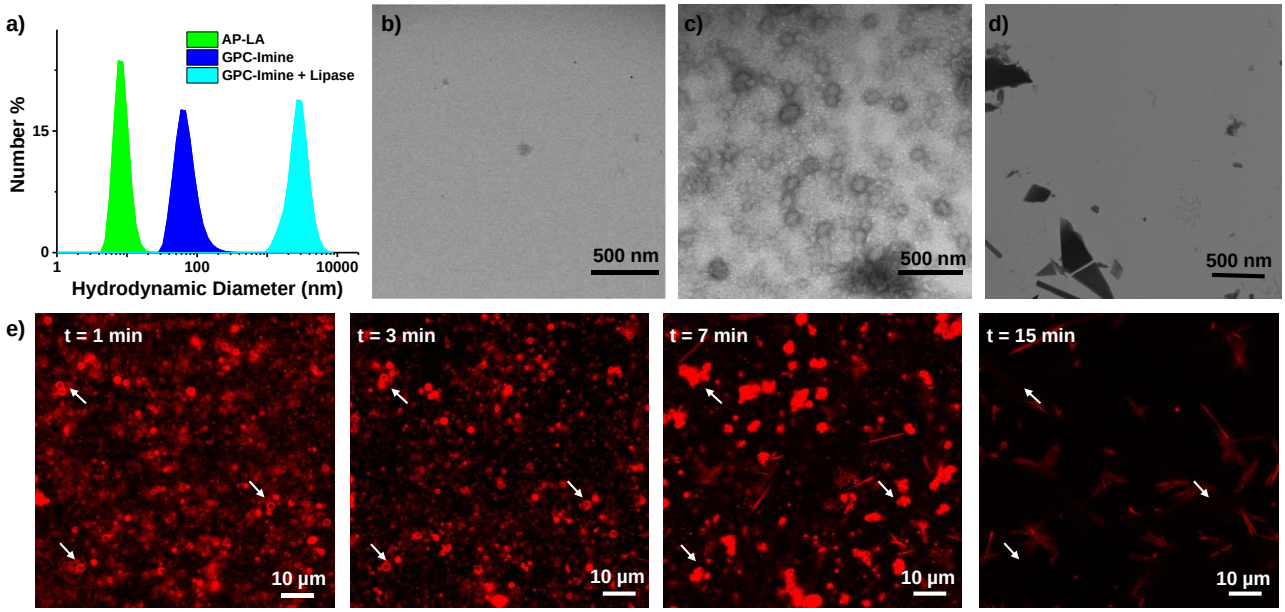


Figure 2. a) DLS data showing the change in hydrodynamic diameter when GPC-CHO and AP-LA react to form GPC-Imine vesicle and upon addition of lipase. TEM micrographs of b) fuel AP-LA; c) GPC-Imine vesicles; d) after addition of lipase to the vesicles. e) Snapshots of confocal video imaging of lipase induced disintegration of vesicles with 2000 U/mL lipase. Conditions: 5 mM GPC-CHO and 15 mM AP-LA.

To understand the supramolecular organization, atomic force microscopy (AFM) was used. Lipid vesicles are known to open into bilayer membranes on mica surface[11]. We observed the formation of ~5 nm thick bilayer membrane (Figure S11), similar to the thickness of biological membranes[12]. The energy minimized structure of GPC-Imine showed a molecular length of ~2.5 nm (Figure S12), indicating that 5 nm is indeed from a bilayer structure. In solution and real-time visualization of these structures using confocal fluorescence microscopy showed complete disintegration of individual vesicles into crystalline structures within 15 minutes. It confirms that initially GPC-Imine forms vesicles that degrade upon enzymatic hydrolysis to generate crystalline LA as waste (Figure 2e, SI video). Although confocal is limited to the visualization of large structures, it confirms the structural transformation in solution.

We have, thus, demonstrated two independent processes: a) anabolic formation of GPC-Imine vesicle, and b) catabolic lipase-catalyzed hydrolysis and disintegration of GPC-Imine vesicle. Next, for simultaneous formation and disintegration of the vesicle, GPC-CHO, AP-LA, and lipase were premixed initially (Figure 3a, Figure S13). *E.g.*, with 400 U/mL of lipase, we observed a fast formation of GPC-Imine within 10 min, followed by a slow hydrolysis in 100 min. This confirms the autonomous formation and degradation of GPC-Imine, resulting in transient lipid vesicles with a specific lifetime. Furthermore, increasing the concentration of lipase resulted in shorter lifetime of the vesicles (> 2 hrs to 20 min for complete hydrolysis), resulting in temporal control (Figure S14).

Finally, we reactivated the system by adding a fresh batch of AP-LA fuel after the first cycle of formation and degradation (60 min), which resulted in regeneration of the GPC-Imine followed by hydrolysis with lipase (Figure 3b, Figure S15). However, damping was observed with each cycle, presumably due to the accumulation of waste, which is common in such closed systems.

Altogether, we have demonstrated the formation of GPC-Imine transient vesicles whose lifetime can be tuned by changing enzyme concentration and the self-assembly can be re-activated by adding fresh fuel. It's worth noting that since lipase naturally resides within the hydrophobic membrane[13], it does not react neither with the fuel nor with the GPC-CHO and the selective hydrolysis of the GPC-Imine is facilitated by the formation of vesicle (Figure S20).

One of the features of biology is to maintain their non-equilibrium structures by continuous metabolism and by constantly consuming fuel. However, synthetic analogs of non-equilibrium steady state (NESS) are seldom reported due to their chemical complexity[3e, 14]. We aimed to sustain the vesicle by maintaining the concentration of GPC-Imine in its NESS by initially adding an excess of fuel (Figure 3c). Thus, we probed the change in the concentration of GPC-Imine using absorbance over time (Figure 3d, AP-LA 9 mM). We observed a plateau for up to 75 min, indicating a NESS. During NESS, the concentration of GPC-Imine and, consequently, the self-assembly is maintained even if the individual lipid (GPC-Imine) is constantly degraded and replaced by new ones. Additionally, increasing the fuel concentration at fixed lipase concentration and vice versa resulted in temporal control of the NESS vesicles from 5 min to 75 minutes and 105 min to 10 min, respectively (Figure 3d-e, Figure S21). Thus, we have demonstrated that our chemically fueled vesicles can be sustained in their NESS with easily controllable lifetime (Figure 3f). Since ours is a closed system, the NESS is referred only w.r.t. the concentration of vesicle-forming GPC-Imine.

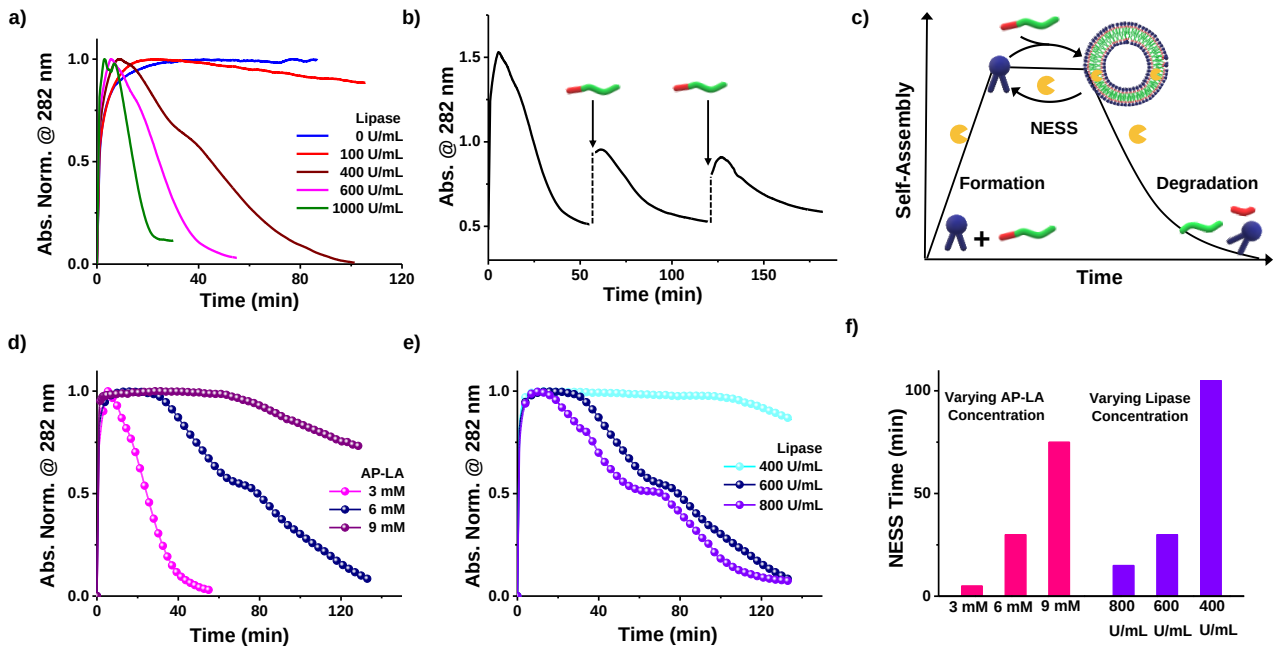


Figure 3. a) Time-dependent absorbance changes in presence of lipase showing the autonomous formation and degradation of GPC-Imine resulting in transient lipid vesicles. b) Refueling the system with fresh batch of AP-LA fuel after first cycle with 1 mM GPC-CHO, 3 mM AP-LA, 600 U/mL lipase. c) Schematic showing the GPC-Imine in NESS. Absorbance changes showing NESS in GPC-Imine concentration d) by varying AP-LA and fixed lipase concentration at 600 U/mL, e) by varying lipase and fixed AP-LA concentration (6 mM). f) Histogram showing the temporal control of the NESS obtained from d), e). Conditions: 1 mM GPC-CHO. Small shoulder observed in b (40 min), d (70 min), e (70 min) are due to precipitation of lauric acid crystals (Figure S22).

It is to be highlighted that in our design, the vesicles are formed by simply mixing the water-soluble precursors (GPC-CHO+AP-LA), leading to the *in situ* formation of PhL GPC-Imine that self-assembles into vesicles of controlled dispersity (PDI = 0.221). Additionally, our nanovesicles, with an average size of approx. 65 nm, is optimal for drug delivery applications. In contrast, traditionally, vesicles formed with PhL require complex methods like film rehydration, solvent switch, etc., to result in polydisperse, multilamellar vesicles that need further extrusion procedures to obtain desirable size[15]. Thus, our vesicle design is highly advantageous which could be due to the *in situ* formation of the PhL GPC-Imine, which self-assembles into uniform vesicles. Therefore, we aimed to demonstrate the application of our chemically fueled vesicles for dynamically regulated cargo release. We have chosen Nile red as a model hydrophobic cargo, which was encapsulated within the hydrophobic membrane of the GPC-Imine vesicle by simple mixing (Figure S23).

The encapsulation of Nile red within the vesicle was characterized by enhanced fluorescence, and the release was monitored by probing the decrease in fluorescence[16]. We observed that GPC-Imine vesicles in their equilibrium state (i.e., without lipase) did not release the cargo, as seen by no change in fluorescence, confirming the impermeable nature of the vesicle (Figure 4). However, in the presence of lipase, the chemically fueled GPC-Imine vesicle showed a continuous decrease in fluorescence intensity, indicating cargo (Nile red) release. Furthermore, increasing the lipase concentration from 400 to 1000 U/mL resulted in faster release kinetics. Thus, we have demonstrated that the fuel-driven vesicles can be used to release the hydrophobic dye, where the release kinetics can be regulated by the rate of enzymatic reaction.

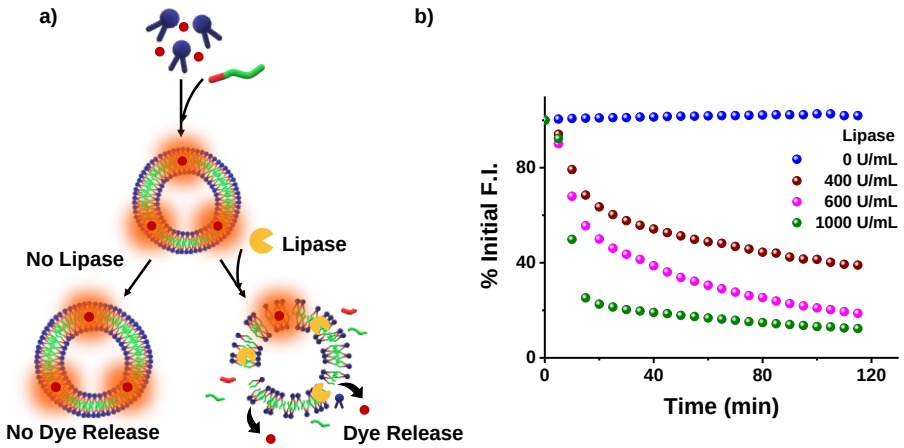


Figure 4. a) Schematic showing the encapsulation of hydrophobic cargo, Nile Red, and its release in presence and absence of lipase. b) Release profile of hydrophobic Nile Red from the chemically fueled vesicles. Conditions: 1 mM GPC-CHO, 3 mM AP-LA and 0.25 mM Nile red.

In summary, we have presented a fuel-driven dissipative self-assembly of PhL into transient vesicles with a controllable lifetime. *In situ* formation of PhL through an imine bond with fuel and lipase-catalyzed ester hydrolysis led to the anabolic formation and subsequent catabolic degradation of vesicles, respectively. This resulted in transient vesicles whose lifetime could be easily tuned by varying the concentration of the fuel or the lipase. Most interestingly, the uniformly sized ~65 nm vesicles were formed autonomously by simply mixing the precursors, therefore avoiding the traditional lengthy

process of film rehydration, membrane extrusion etc[15]. These optimally sized vesicles were used to encapsulate a model cargo and the release kinetics could be dynamically regulated by the enzyme concentration. Thus, our biomimetic design of metabolic PhL into uniform vesicles, combined with the ease of vesicle formation and controlled cargo release, makes it an ideal candidate for adaptive nanomedicine. Furthermore, since lipase is overexpressed in many inflammatory diseases, our lipase-regulated cargo release will be suitable for targeted therapy.

Supporting Information

The authors have cited additional references within the Supporting Information.

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Keywords: Supramolecular Chemistry • Phospholipids • Vesicles• Systems Chemistry • Transient Assembly

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