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Design and Synthesis of Out-of-Equilibrium Phospholipid Vesicles

Akhil Venugopal



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Doctoral Thesis

Design and Synthesis of Out-of-Equilibrium Phospholipid Vesicles

Disseny i síntesi de vesícules fosfolípides fora d'equilibri

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Directors: Dr. Mohit Kumar & Prof. Giuseppe Battaglia



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Disseny i síntesi de vesícules fosfolípides fora d'equilibri

Memòria presentada per optar al grau de doctor per la

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Declaration of Authorship

I, Akhil Venugopal, hereby declare that the work presented in this thesis, entitled “Design and Synthesis of Out-of-Equilibrium Phospholipid Vesicles”, is the result of original investigations carried out by me at the Molecular Bionics Lab, Institute for Bioengineering of Catalonia (IBEC), under the supervision of Dr. Mohit Kumar and Prof. Giuseppe Battaglia. All sources of information and material derived from the work of others have been appropriately cited and referenced within the thesis.

Barcelona

30 September 2025

A handwritten signature in black ink, appearing to read 'Akhil Venugopal', written diagonally across the page.

Akhil Venugopal

Acknowledgements

“ഓരോ മനുഷ്യനും, തന്റെ ചുറ്റുമുള്ളവരുടെ സ്നേഹത്തിലും സ്വാധീനത്തിലും രൂപപ്പെടുന്നു.”

(Every human being is shaped by the love and influence of people around them)

Human beings are not solitary creatures, we grow, change, and evolve through the presence of others. No one walks alone; each life is shaped by the countless souls we meet, the voices we hear, and the hands that guide us along the way. This thesis, though written by me, carries within it the love, support, and wisdom of many. Without their patience, encouragement, and unwavering belief in me, I would not have reached this page.

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Thank you all for being a part of my journey and shaping me!

Abbreviations

AFM	Atomic force microscopy
AMP	Adenosine 5'-monophosphate
AP	Aminopropanol
ATP	Adenosine 5'-triphosphate
CAC	Critical aggregation concentration
CDCl ₃	Chloroform-d
CLSM	Confocal laser scanning microscopy
CPP	Critical packing parameter
CuAAC	Cu-catalyzed alkyne–azide cycloaddition
CytC	Cytochrome C
D ₂ O	Deuterium oxide
DCC	N, N'-dicyclohexylcarbodiimide
DCM	Dichloromethane
DLS	Dynamic Light scattering
DMAP	4-(N, N-dimethylamino) pyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d ₆	Dimethyl sulfoxide-d ₆
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
GOx	Glucose oxidase
GPC-CHO	Phosphorylcholine aldehyde
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPLC	High performance liquid chromatography
HPNPP	2-Hydroxypropyl p-nitrophenyl phosphate
HPTS	8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt
HRMS	High-resolution mass spectrometry

HRP	Horseradish peroxidase
iSCAT	Interferometric scattering microscopy
LA	Lauric acid
mM	Millimolar
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OOE	Out-of-Equilibrium
PDI	Polydispersity index
SNAr	Nucleophilic aromatic substitution reaction
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
UV light	Ultraviolet light
UV/Vis	Ultraviolet/Visible
μM	Micromolar

Abstract

This thesis focuses on the design, synthesis and application of out-of-equilibrium (OOE) phospholipids constructed from simple, water-soluble building blocks. Phospholipids are fundamental amphiphiles that spontaneously organize into supramolecular membranes, providing the structural and functional framework of all living cells. Life itself depends on the chemical and physical transformation of diverse biomolecules such as proteins, nucleic acids, carbohydrates, and lipids. Yet these transformations are orchestrated within highly organized compartments defined by phospholipid bilayers of only a few nanometers thick. These membranes are far more than passive barriers; they enable selective transport, spatial organization, and continuous remodeling, thereby supporting essential processes such as signaling, metabolism, and replication across a range of length scales, from nanometers to entire cellular architectures. In this thesis, I draw inspiration from these natural architectures to investigate how OOE processes of living cells can be incorporated into the design of synthetic phospholipid vesicles to achieve life-like features. By integrating metabolic-like fueling strategies, the work demonstrates how artificial membranes can be generated, remodeled, and functionally sustained, offering insights into the design of adaptive systems for applications in nanomedicine. In essence, these dynamic vesicles follow the philosophy of being “*of biology, by biology, for biology*,” as they are inspired by cellular processes, operate through life-like principles, and aim to engage more effectively with biological systems. Hence, this thesis aims to push systems chemistry towards biology, focusing on how fuel-driven reaction networks can generate and remodel phospholipid compartments that are central to life itself.

Chapter 1 provides a comprehensive literature survey on vesicular self-assembly under OOE conditions. It begins with the basic principles of chemically fueled OOE cellular membranes, highlighting how biological processes rely on continuous energy dissipation to maintain dynamic structures and functions. The chapter then briefly reviews synthetic vesicles under equilibrium conditions, which have long served as models for membrane formation but remain inherently static in nature, i.e., under thermodynamic control. Here, we will mainly highlight vesicular assemblies that emerge from in situ lipid-forming systems in aqueous media, generated through chemical reactions between simple water-soluble precursors. The discussion then shifts to recent advances in fuel-driven strategies for the formation of vesicles under OOE, covering both 1) non-phospholipid systems that showcase the breadth of dissipative chemistry and 2) phospholipid-based systems that most closely resemble natural membranes. Together, these studies set the stage for the design of synthetic lipid systems with adaptive, life-like features and thereby laying the conceptual groundwork for the experimental work described in the following chapters.

Chapter 2 describes the chemically fueled, OOE self-assembly of synthetic phospholipids into vesicles inspired by the metabolic processes in natural membranes. In living systems, phospholipid turnover is continuously regulated by metabolic cycles that consume fuel to regulate lipid synthesis and degradation, thereby controlling membrane properties. Drawing inspiration from this principle, we developed a minimal chemical reaction network in which simple, water-soluble precursors undergo fuel-driven transformation into double-tailed phospholipids that spontaneously self-assemble into vesicles. Crucially, these assemblies are transient; their lifetime can be tuned from minutes to hours by modulating the kinetic competition between anabolic-like formation and enzymatic catabolism. Beyond their fundamental significance, these nanoscale vesicles offer translational potential through the encapsulation and regulated release of cargo molecules under OOE conditions, opening avenues for adaptive nanomedicine and controlled drug delivery. This chapter represents a cornerstone of the thesis, establishing that phospholipid-based vesicles can indeed be sustained and controlled by artificial metabolic cycles.

Chapter 3 extends this concept to the membrane formation and OOE membrane remodeling of phospholipid vesicles. Here, we systematically varied the hydrophobic tails of lipid precursors and uncovered how tail length directly influences membrane formation. Longer hydrophobic chains accelerated vesicle formation, while shorter chains slowed the process. We also allowed the system to compete and self-select by mixing different fuels, thereby revealing the inherent preferences of lipid assembly. Under equilibrium conditions, the system always converged to a mixed population of asymmetric lipids, giving rise to uniquely shaped compartments. In contrast, under dissipative conditions, kinetic asymmetries reshaped the outcome where fast forming lipid species were rapidly depleted, biasing the system towards distinct lipid populations. This chapter highlights, for the first time, how simple chemical design coupled with non-equilibrium conditions can emulate the adaptive remodelling of biological membranes.

Finally, **Chapter 4** presents an overall summary of the work. It also, provides an outlook on how these findings can be applied toward the generation of more integrated, artificial cellular systems. By combining lipid metabolism with other synthetic reaction networks, it may become possible to design compartments that grow, divide, and remodel in response to external stimuli. Such advances would not only shed light on the chemical origins of life but also pave the way for engineering soft, adaptive materials for nanomedicine. Also, this chapter discusses the challenges of this field, more specifically related to the characterization of dynamic supramolecular events in solution, and highlights the importance of the real-time, in situ visualization of these dynamic supramolecular systems.

Keywords: supramolecular chemistry, phospholipids, vesicles, systems chemistry, out-of-equilibrium assembly.

Resum

Aquesta tesi se centra en el disseny, la síntesi i l'aplicació de fosfolípids fora de l'equilibri (OOE) construïts a partir de blocs de construcció simples i solubles en aigua. Els fosfolípids són amfífils fonamentals que s'organitzen espontàniament en membranes supramoleculares, proporcionant l'estructura i el marc funcional de totes les cèl·lules vives. La vida mateixa depèn de la transformació química i física de diverses biomolècules com proteïnes, àcids nucleics, carbohidrats i lípids. Tot i així, aquestes transformacions es duen a terme dins de compartiments altament organitzats definits per bicapas fosfolipídiques de només uns pocs nanòmetres de gruix. Aquestes membranes són molt més que simples barreres passives; permeten el transport selectiu, l'organització espacial i la remodelació contínua, donant suport a processos essencials com la senyalització, el metabolisme i la replicació a escales que van dels nanòmetres fins a arquitectures cel·lulars senceres. En aquesta tesi, m'inspiro en aquestes arquitectures naturals per investigar com els processos fora de l'equilibri de les cèl·lules vives poden incorporar-se en el disseny de vesícules fosfolipídiques sintètiques per obtenir característiques semblants a la vida. Integrant estratègies de subministrament de combustible semblants al metabolisme, el treball demostra com es poden generar, remodelar i mantenir funcionalment membranes artificials, aportant idees per al disseny de sistemes adaptatius amb aplicacions en nanomedicina. En essència, aquestes vesícules dinàmiques segueixen la filosofia de ser «de la biologia, per la biologia i per a la biologia», ja que s'inspiren en processos cel·lulars, operen mitjançant principis semblants als de la vida i pretenen interactuar de manera més eficaç amb els sistemes biològics. Així doncs, aquesta tesi pretén portar la química de sistemes cap a la biologia, centrant-se en com les xarxes de reaccions impulsades per combustible poden generar i remodelar compartiments fosfolipídics que són centrals per a la vida mateixa.

Capítol 1 ofereix una revisió exhaustiva de la bibliografia sobre l'autoassemblatge vesicular en condicions fora de l'equilibri (OOE). Comença amb els principis bàsics de membranes cel·lulars químiques fora de l'equilibri, destacant com els processos biològics depenen de la dissipació contínua d'energia per mantenir estructures i funcions dinàmiques. El capítol revisa breument les vesícules sintètiques en condicions d'equilibri, que durant molt de temps han servit de models per a la formació de membranes, però que romanen intrínsecament estàtiques sota control termodinàmic. Aquí, ens centrarem sobretot en les estructures vesiculars que emergeixen de sistemes in situ de formació de lípids en medis aquosos, generats mitjançant reaccions químiques entre precursors senzills i solubles en aigua. Aquest capítol també discuteix avenços recents en estratègies impulsades per combustible per a la formació de vesícules en condicions OOE, cobrint tant 1) sistemes no fosfolipídics que mostren l'amplitud de la química dissipativa com 2) sistemes basats en fosfolípids que s'assemblen més a les membranes naturals. Junts, aquests estudis preparen

el terreny per al disseny de sistemes lipídics sintètics amb característiques adaptatives semblants a les de la vida, establint les bases conceptuals per al treball experimental descrit als capítols següents.

Capítol 2 descriu l'autoassemblatge químicament impulsat i fora de l'equilibri de fosfolípids sintètics en vesícules inspirades en processos metabòlics de membranes naturals. En sistemes vius, el recanvi de fosfolípids està regulat contínuament per cicles metabòlics que consumeixen combustible per controlar la síntesi i la degradació lipídica, modulant així les propietats de la membrana. Inspirant-nos en aquest principi, vam desenvolupar una xarxa química mínima en la qual precursors senzills i solubles en aigua es transformen, mitjançant combustible, en fosfolípids de doble cua que s'autoassemblen espontàniament en vesícules. Crucialment, aquests conjunts són transitoris; la seva vida mitjana pot ajustar-se de minuts a hores modulant la competència cinètica entre la formació anabòlica i el catabolisme enzimàtic. Més enllà del seu significat fonamental, aquestes vesícules a nanoescala ofereixen potencial translacional mitjançant l'encapsulació i l'alliberament regulat de càrregues sota condicions OOE, obrint vies per a nanomedicina adaptativa i administració de fàrmacs controlada. Aquest capítol representa una pedra angular de la tesi, establint que les vesícules basades en fosfolípids poden ser sostingudes i controlades mitjançant cicles metabòlics artificials.

Capítol 3 amplia aquest concepte a la formació de membranes i la remodelació fora de l'equilibri de vesícules fosfolipídiques. Aquí, vam variar sistemàticament les cues hidrofòbiques dels precursors lipídics i vam descobrir com la longitud de la cua influeix directament en la formació de membranes. Les cadenes hidrofòbiques més llargues van accelerar la formació de vesícules, mentre que les més curtes van alentir el procés. També vam permetre que el sistema competís i s'autoseleccionés mitjançant la barreja de diferents combustibles, revelant així les preferències inherents de l'assemblatge lipídic. En condicions d'equilibri, el sistema sempre convergia cap a una població mixta de lípids asimètrics que donaven lloc a compartiments amb formes úniques. En canvi, en condicions dissipatives, les asimetries cinètiques van remodelar el resultat, ja que les espècies de formació ràpida es van esgotar ràpidament, inclinant el sistema cap a poblacions lipídiques distintes. Aquest capítol destaca, per primera vegada, com un disseny químic senzill combinat amb condicions fora de l'equilibri pot emular la remodelació adaptativa de membranes biològiques.

Finalment, el Capítol 4 ofereix una perspectiva sobre com aquests resultats poden aplicar-se a la generació de sistemes cel·lulars artificials més integrats. Combinant el metabolisme lipídic amb altres xarxes de reaccions sintètiques, podria ser possible dissenyar compartiments que creixin, es divideixin i es remodelin en resposta a estímuls externs. Aquests avenços no només aportarien llum sobre els orígens químics de la vida, sinó que també obririen el camí per enginyar materials tous i

adaptatius per a la nanomedicina. Aquest capítol també discuteix els reptes d'aquest camp, especialment relacionats amb la caracterització d'esdeveniments supramoleculars dinàmics en solució, i subratlla la importància de la visualització en temps real i *in situ* d'aquests sistemes supramoleculars dinàmics.

Paraules clau: química supramolecular, fosfolípids, vesícules, química de sistemes, autoassemblatge fora de l'equilibri.

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Chapter 1: Introduction

1.1 Prelude

Self-assembly, where simple molecular or structural elements give rise to complex, regulated, and functional architectures, is ubiquitous in nature. Nature reveals collective order at every level, from the coordinated movement of bird flocks to the intricate folding of proteins and the organization of ribosomes where structures reorganize themselves through self-assembly.^[1] Molecular self-assembly arises when molecules spontaneously organize into higher order architectures through predominantly weak, non-covalent interactions to minimize their free energy.^[2] Common forces driving this process include van der Waals interactions, electrostatic (Coulomb) interactions, hydrogen bonding, hydrophobic effects, π -electrons related interactions such as π - π stacking or cation- π interactions and coordination bonds. Through these interactions, a system transitions from a less ordered state to a more ordered configuration. For decades, supramolecular chemistry has taken inspiration from these examples, creating synthetic assemblies that form spontaneously under the guidance of thermodynamic stability.^[3] In such systems, the assembled state lies at a lower free energy than the individual components (Figure 1.1a), making these structures highly stable once formed. Their robustness makes them ideal for biological roles that demand persistence and reliability. The reliability and stability of equilibrium assemblies have been exploited in countless contexts. Thermodynamically stable nanostructures have provided the basis for new equilibrium-based materials in organic electronics, drug delivery, sensing, and cosmetics, among other areas.^[4-6]

However, stability alone is not enough when function requires rapid and precise spatial or temporal control as seen in living systems. In Nature, molecular assemblies are rarely static. Instead, they are highly dynamic which assemble (anabolism) and disassemble (catabolism) continuously, by consuming the chemical energy stored in activated molecules, or fuels, to drive assemblies that are not thermodynamically favoured.^[7] These fuel-dependent assemblies exist only while the energy source is available, with the lifetime of the assemblies directly dependent on the consumption of fuel. Once the fuel is consumed and converted into waste products, the system relaxes back to its unassembled building blocks, as the waste can no longer sustain the ordered state (Figure 1.1b). Nature relies on such out-of-equilibrium (OOE) assemblies whenever precise spatial and temporal control is needed. The cytoskeleton provides a classic example where proteins such as tubulin form dynamic polymers that are powered by GTP.^[8] These polymers constantly build and break down, giving cells both strength and flexibility. Without GTP, the polymers fall apart, and the cell loses its structural integrity. This dynamic instability captures the essence of energy-driven self-assembly where growth and shrinkage occur in a tightly regulated cycle that is vital for processes such as cell

division, motility, and intracellular transport (Figure 1.1c). What sets fuel-driven assemblies apart from equilibrium assemblies is that their behaviour depends mainly on the kinetics of anabolic and catabolic reactions, not just the free energy of the final structure. As long as fuel is available, the kinetics bias the system toward an organized, OOE state. Once the fuel is consumed, the assembly collapses.

Over the past decade, researchers have increasingly sought to design OOE supramolecular systems that mimic the transient, adaptive behaviour of biological assemblies. Instead of aiming for maximum stability, the goal is now to create materials that form, persist, and decay on demand, with lifetimes encoded by fuel turnover. This transition represents the birth of chemically-fueled supramolecular chemistry, a field that explicitly embraces kinetics, feedback, and energy flux as design parameters.

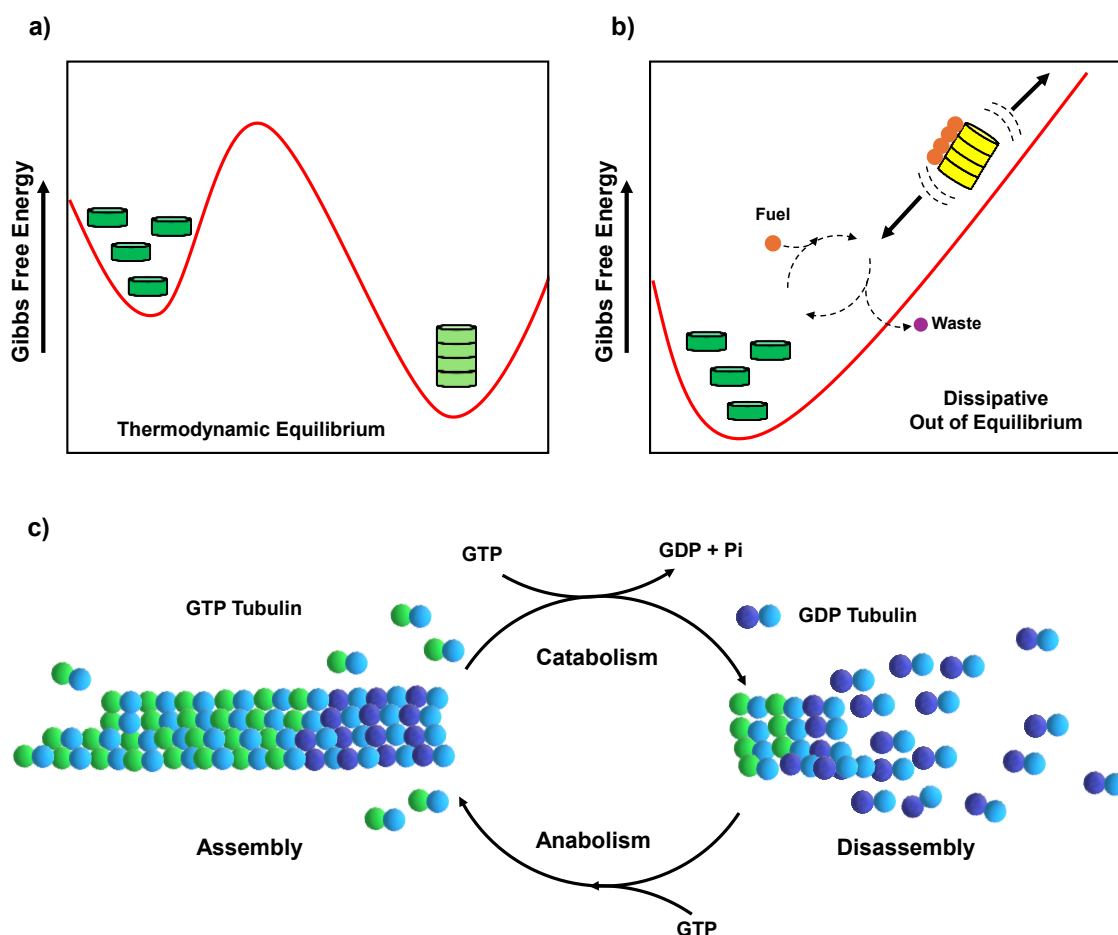


Figure 1.1. Energy diagrams showing a) equilibrium self-assembly where the assembled state resides at a global minimum, b) OOE self-assembly where continuous presence of the fuel drives the formation of the assembly and maintains the presence of the assembled state. As the fuel is consumed to produce waste, the building blocks are restored. c) Schematic showing the GTP induced dynamic polymerization of cytoskeleton proteins.

One of the initial and seminal works displaying synthetic OOE self-assembly was reported by van Esch, Eelkema and co-workers.^[9-10] The authors used a chemical reaction cycle to gain access to transient self-assembling active materials. They synthesized a dibenzoyl-L-cystine bearing two carboxylate groups (**2a**), that are unable to self-assemble due to the repulsive interactions of the charge at neutral pH (Figure 1.2a). Addition of a standard methylating agent, dimethyl sulphate as a chemical fuel into the medium converted the acids into the corresponding esters (**2b**), thereby neutralizing the charge repulsion. The esters spontaneously self-assembled into fibrillar structures resulting in a self-supporting gel. The spontaneous hydrolysis of esters under alkaline conditions provided a built-in deactivation step, ensuring that the system returned to equilibrium after fuel consumption. By carefully tuning the concentrations of fuel and base, the authors created a OOE cycle in which high-energy building blocks assembled, dissipated energy, and disassembled over time. Microscopic studies revealed striking dynamic behaviour, including growth and shrinkage of fibres from their ends and oscillations in fibre length, hallmarks of a system maintained under OOE conditions (Figure 1.2b). This work provided one of the clearest early proofs that supramolecular assemblies can be programmed to exist only under fueling conditions, thereby mimicking the transient, energy-dependent organization observed in living systems. It laid the conceptual foundation for the field of OOE supramolecular chemistry, where structure and function emerge not from static thermodynamic stability, but from continuous energy flux and regulated turnover.

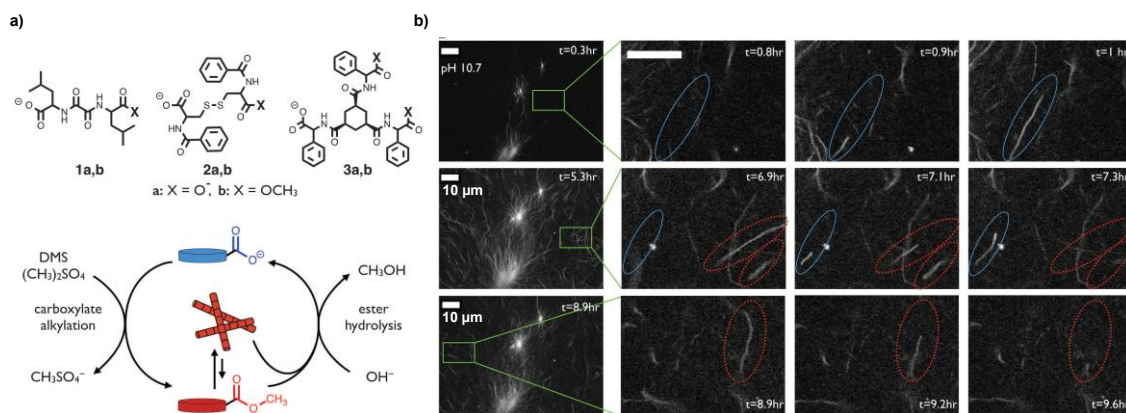


Figure 1.2. a) Structures of the building block (*above*); Carboxylate building blocks are methylated by the fuel dimethyl sulphate, leading to fibre formation. Base present in the medium hydrolyses the esters back to the acid resulting in disassembly (*below*). b) Confocal images over time, showing the dynamic behaviour (growth, shrinkage and overlap) of fibers during a reaction cycle. Fibers undergoing elongation are highlighted with blue circles, while those undergoing shortening are marked with red circles. Figures adapted with permission from reference ^[9]. Copyright 2015 The American Association for the Advancement of Science (AAAS).

On this foundation, numerous efforts have sought to diversify and expand the scope of OOE self-assembly. This progress has given rise to a wide range of temporally programmed OOE assemblies, spanning small organic molecules, polymers, nanoparticles, and droplets.^[11-16] A variety of

strategies have been developed to drive and regulate these systems, including chemical reaction networks, enzyme-mediated transformations, light-responsive chemistries, and continuous flow setups.^[17-22] Importantly, these approaches have demonstrated that temporal control can be harnessed to realize functions reminiscent of living systems, such as fuel-regulated catalysis, self-erasing inks, adaptive photonic and electronic responses, and even rudimentary self-replication.^[23-29] Each of these examples^[30-39] illustrates the growing toolbox of strategies available for designing synthetic systems that are no longer defined by thermodynamic minima, but by kinetically controlled, fuel-driven cycles, imparting synthetic systems with lifetimes, dynamics, and adaptive properties that equilibrium assemblies cannot achieve.

The delicate balance between activation and deactivation defines the assembly's structure, its stability, and its lifetime. Importantly, because the rate constants of these processes can differ in the assembled and unassembled states, nonlinear dynamics and emergent behaviours often arise. Hence, the hallmark features of living systems like transience, adaptability, feedback, and emergence are now being captured in synthetic supramolecular systems.

Today, the field of OOE supramolecular chemistry is rapidly maturing. Perhaps most importantly, these systems provide model platforms to ask deep questions about the origins of life like how simple molecules, under continuous energy input, might have given rise to the first self-organizing, adaptive structures. In summary, supramolecular chemistry has progressed from designing stable, equilibrium assemblies toward building dynamic, fuel-driven systems that more closely mirror the behaviour of biology. By incorporating energy dissipation and kinetics into design, chemists are opening new possibilities for materials that do not simply exist, but emerge, act, and dissipate in controlled timeframes.

Most of the systems operate under OOE conditions reported so far have largely given rise to one-dimensional nanofibers and two-dimensional nanosheets, often inspired by the dynamic assembly and disassembly of microtubules in cells. Yet it is worth noting that the cellular membrane itself constitutes an OOE system, where lipid synthesis and turnover occur continuously through cyclic metabolic processes. Central to this is the self-assembly of phospholipids into bilayer membranes, which create dynamic boundaries that compartmentalize chemical potential and sustain metabolism, arguably one of the most fundamental hallmarks of life.

Before discussing recent advances in the OOE formation of vesicular nanostructures, it is essential to first appreciate the conceptual and practical foundation laid by equilibrium vesicle systems, that provide both the inspiration and the experimental framework for the emerging energy-dissipative architectures. It is important to note that, in the context of this thesis, the term equilibrium vesicles does not imply that the vesicles are at thermodynamic equilibrium. Rather, these are kinetically

trapped assemblies such as traditional lipid vesicles which appear stable over experimental timescales despite not being in true thermodynamic equilibrium.

1.2 Vesicles as Supramolecular Assemblies

Inspired by the central biological phenomena of compartmentalization, chemists have long sought to construct synthetic analogues of compartments. Vesicles represent a critical class of supramolecular assemblies for mimicking cellular compartmentalization. As closed bilayer structures, vesicles not only isolate and concentrate chemical reactions but also provide platforms for controlled exchange with the environment. At the molecular level, vesicle formation is driven by the amphiphilic nature of molecules, where hydrophilic headgroups and hydrophobic tails spontaneously organize in aqueous environments to minimize interfacial free energy (Figure 1.3a). Among these, phospholipids represent a major class of amphiphiles comprising of phosphocholine headgroup linked to two fatty acid tails with varying length and degree of saturation (**4** and **5**). This structural diversity directly affects the critical packing parameter (CPP), a geometric factor that relates headgroup area, tail volume, and chain length, thereby dictating whether amphiphiles assemble into micelles, bilayers, or inverted phases (Figure 1.3b-c).

Their defining feature is the bilayer arrangement, where hydrophilic headgroups interface with the aqueous medium while hydrophobic tails are sequestered within the membrane interior. This amphiphilic organization mirrors the architecture of cellular membranes and provides a minimal yet powerful platform for studying compartmentalization. These vesicles can encapsulate molecular cargo, support selective exchange across the membrane, and even host biochemical reactions, thereby functioning as simplified models of living cells. Their structural resemblance to biological membranes has made them essential in protocell research, drug delivery, and the design of biochemical microreactors.

1.3 Equilibrium in situ vesicles

One of the main advantages of equilibrium vesicles lies in their relative stability and reproducibility. Because they assemble spontaneously under thermodynamically favourable conditions, they can be prepared with comparative ease and maintained over extended timescales. These features have established them as indispensable tools in supramolecular chemistry and synthetic biology, where they continue to serve as testbeds for exploring fundamental principles of organization, compartmentalization, and function. While the majority of equilibrium vesicles studied to date rely on the vast repertoire of naturally occurring or synthetically made lipids, fatty acids, or block copolymers, here I want to specifically highlight synthetic systems where vesicular assemblies emerge from in situ chemical reactions between simple precursor molecules. In these designs, one

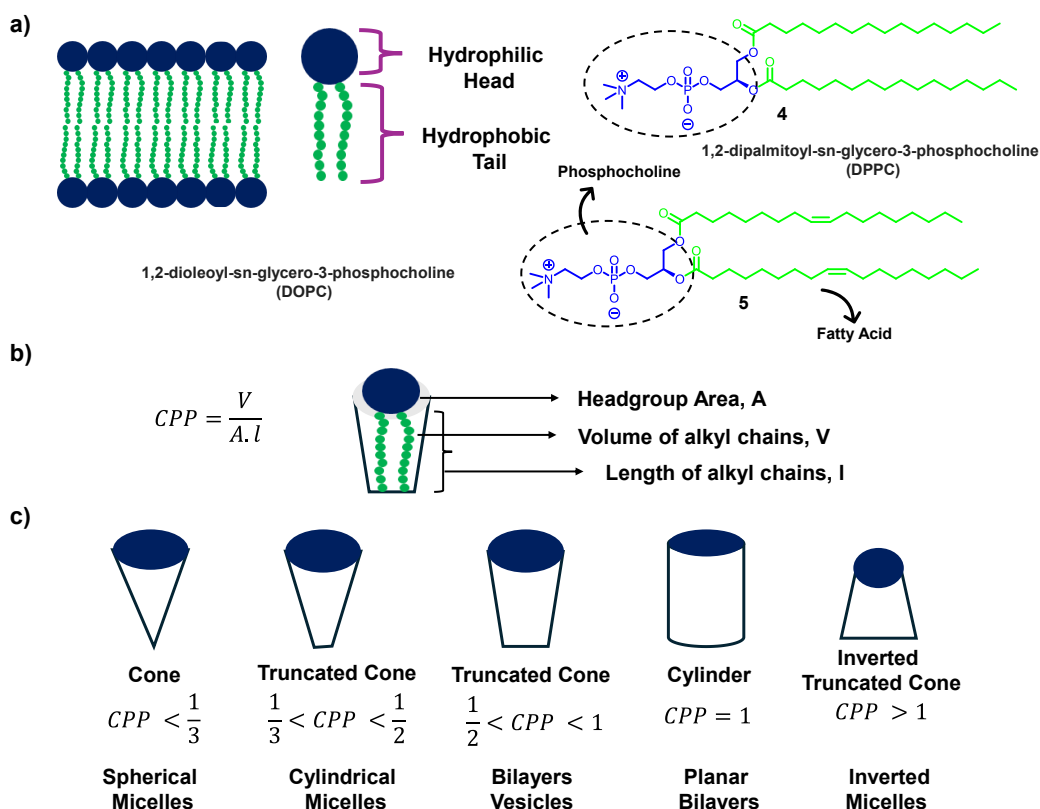


Figure 1.3. a) Schematic and chemical structures of phospholipids showing the hydrophilic head and hydrophobic tail. b) Schematics of the parameters defining the Critical Packing Parameter, CPP. c) Approximate thresholds for the value of CPP for geometry classification of lipids self-assembly.

precursor provides the hydrophilic part and the other the hydrophobic part, together forming amphiphilic molecules that self-assemble into vesicles. They differ from the traditional approach of simply dispersing pre-formed amphiphiles, instead, simple precursors are transformed into amphiphilic products which then self-assemble into stable vesicles under equilibrium conditions. These studies underscore how equilibrium vesicles can be accessed through synthetic routes that generate the amphiphiles on demand.

1.3.1 Non-Phospholipid Vesicles

Dynamic covalent chemistry of imine bonds offers a versatile route for generating vesicular architectures. The facile formation and rapid reversibility of the imine condensation reactions make such assemblies especially attractive for biological applications. van Esch and co-workers have used dynamic imine bond formation between **6** and **7** to create a responsive vesicular system **8** whose formation and disintegration can be regulated reversibly through changing the pH (Fig. 1.4a).^[40] However, the vesicles formed from this system were prone to hydrolysis upon dilution, in contrast to the innate stability of phospholipid membranes. Using the same strategy of reversible imine–aldehyde reaction, Takakura et al.^[41] were able to show a spontaneous structural transformation where micelles reorganized into vesicles. Otto and co-workers^[42] reported the

emergence of vesicular compartments from unconventional surfactants embedded within dynamic combinatorial libraries, where reversible covalent chemistry and self-assembly are directly coupled. Together, these examples confirm that reversible chemistry can guide amphiphiles toward compartmentalization, providing a conceptual link between molecular diversity and the spontaneous emergence of vesicular assemblies.

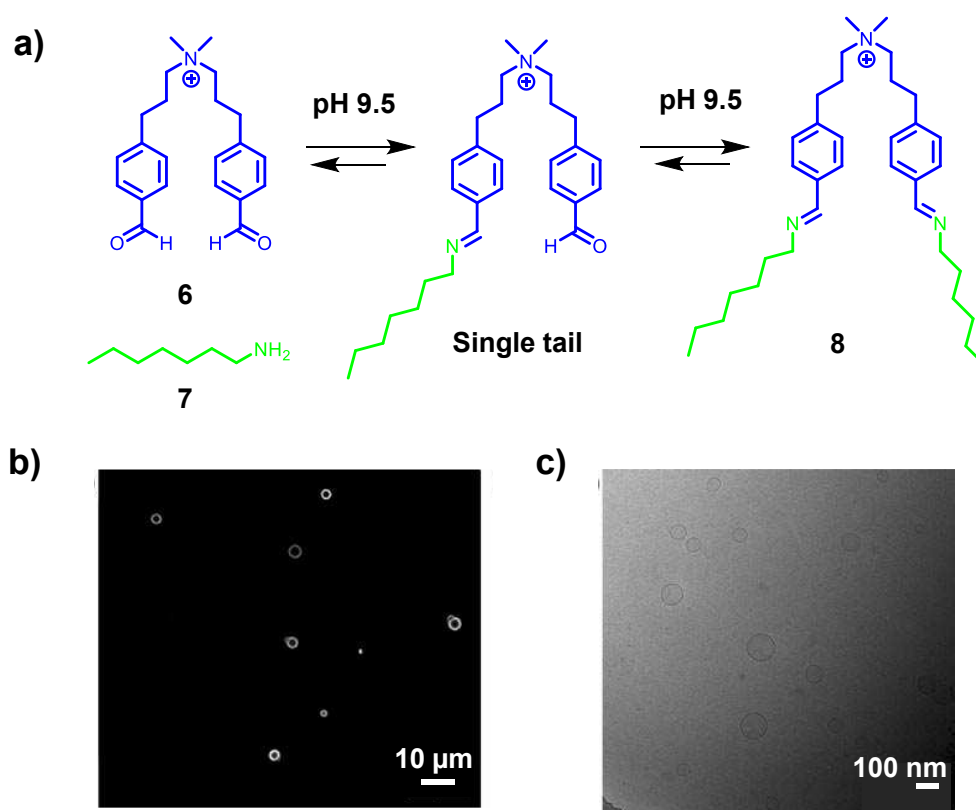


Figure 1.4. a) Chemical structures of the dynamic imine surfactant. b) Confocal images of the vesicles formed. c) Transmission Electron Microscope (TEM) image of the vesicles. Figures adapted with permission from reference ^[40]. Copyright 2011 Wiley-VCH GmbH.

1.3.2 Phospholipid Vesicles

Most of the pioneering work in the in situ synthesis of phospholipids has been carried out by Devaraj and co-workers.^[43] His group has extensively explored the de novo vesicle formation, growth, and reproduction of phospholipids, providing critical insights into the fundamental chemical principles that may have contributed to the origin of life.^[44-46] A large portion of these studies focuses on the formation of phospholipid vesicles under equilibrium conditions, where the resulting bilayer structures serve as model compartments for protocell research. To achieve this, the Devaraj group has developed and applied a range of strategies, mainly enzymatic and chemoenzymatic methods, that are controllable, rapid, selective, and robust for phospholipid generation and subsequent vesicle assembly.^[47-49]

As an alternative to enzyme-based strategies for phospholipid synthesis, they used biomimetic chemistries that bypass biological catalysts altogether, thereby enabling the formation of membranes composed of non-canonical phospholipids (Figure 1.5a-b).^[50-51] For instance, they reported the generation of lipid analogues via dynamic imine bond formation between lysosphingomyelin (**9**) and a variety of aldehydes (**10**) (Figure 1.5a).^[52] The resulting amphiphiles (**11**) were capable of spontaneously assembling into vesicles in aqueous media (Figure 1.5c-d), although a limitation of this approach was the competing micelle formation by the lipid precursor. To address this drawback, the group later introduced a polar headgroup precursor bearing an aminoxy functionality (**12**), which enabled oxime ligation with aldehyde (**13**) chains leading to amphiphilic lipids (**14**, Figure 1.5b).^[53] This modification minimized undesired micelle formation and provided a more robust pathway to vesicle assembly under mild conditions (Figure 1.5e). Bowman and co-workers used the highly efficient thiol-Michael addition reaction to form a functionalized phospholipid through a nucleophile-catalysed reaction between a lysolipid with a primary thiol and an aliphatic acrylate tail.^[54] Although this approach achieved nearly 90% conversion, it required 48 hours under ambient conditions, using a weak nucleophilic catalyst and a stoichiometric reactant mixture.

Among reaction-based strategies, light-controlled in situ formation of phospholipids has also been reported. Light offers a non-invasive and highly controllable stimulus, making it attractive for triggering lipid synthesis under mild conditions. Zhu and co-workers^[55] used photoligation chemistry to convert non-membrane molecules, specifically lysosphingomyelin (**17**) and alkyl substituted 2-nitrobenzyl alcohol derivatives (**15**), into phospholipids (Figure 1.5f). Upon irradiation, 2-nitrobenzyl alcohols are photoconverted into reactive aldehydes (**16**), which undergo selective ligation with the lyso amine to yield phospholipid (**18**) that spontaneously assemble into bilayer vesicles (Figure 1.5g-h). The amphiphilic nature of lysosphingomyelin drives its co-assembly with **16** in aqueous solution, bringing the precursors into close proximity and thereby accelerating the phospholipid formation. Despite its promise, the approach faced several limitations such as the incomplete aldehyde conversion and formation of sheet like aggregates in longer chain **16/17** mixtures.

Devaraj and co-workers introduced a light-gated strategy for constructing phospholipids that respond simultaneously to optical and chemical cues (Figure 1.5i).^[56] Their design featured a cationic bis-aldehyde surfactant headgroup bearing an azobenzene unit (**19**), which undergoes reversible trans-cis photoisomerization under 365 nm and 470 nm light. Condensation of the aldehydes (**20**) with alkyl amines produced amphiphilic imines (**21**) that initially organized into disordered aggregates. Upon irradiation with 365 nm light the trans-azo isomer converted to cis-azo, causing the aggregates to reorganize into giant lamellar vesicles, which subsequently disassembled once the illumination ceased (Figure 1.5j).

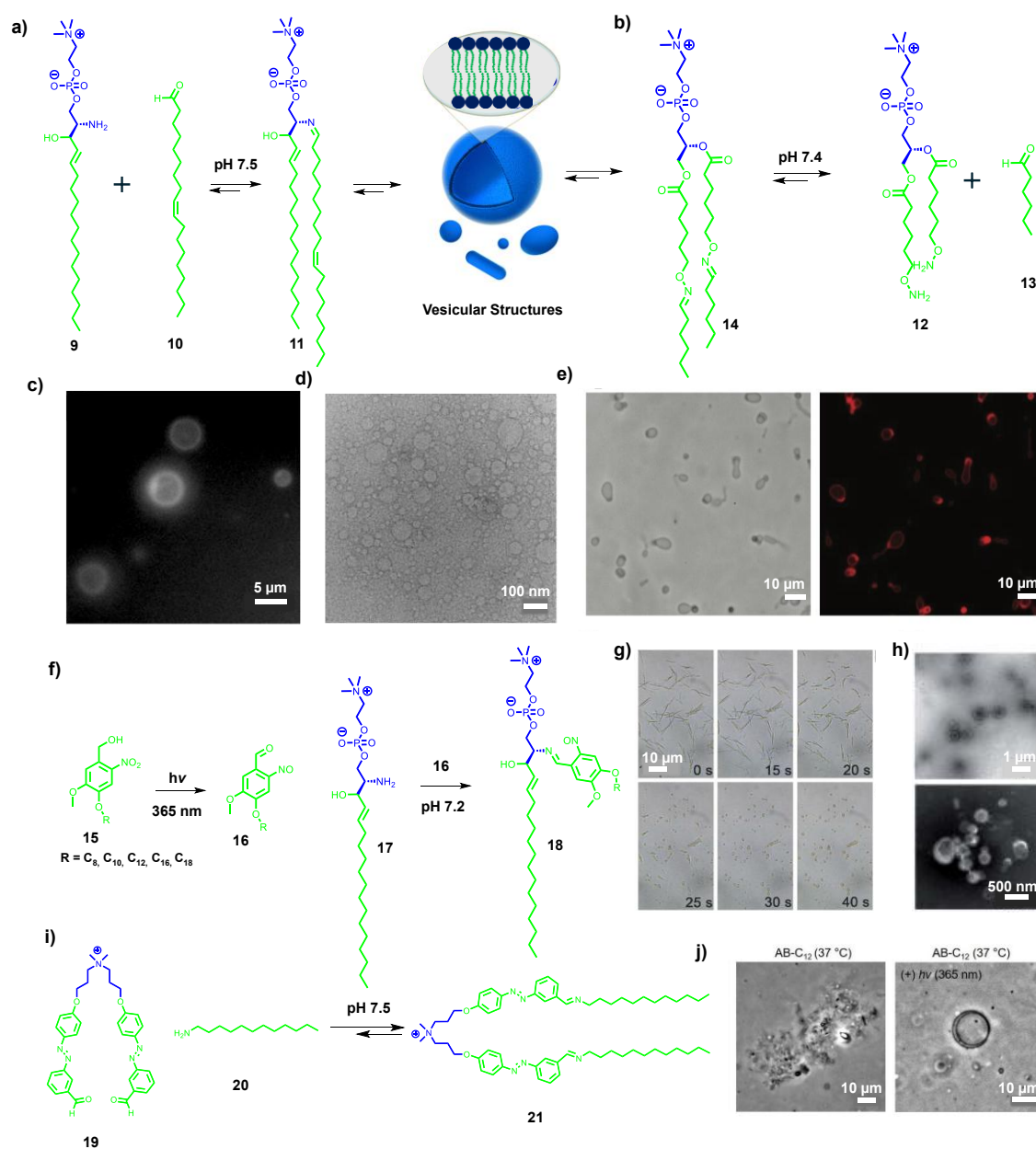


Figure 1.5. a) Generation of vesicles driven by imine formation. b) Generation of vesicles driven by oxime ligation. c) Fluorescence microscopy images of **11**. d) TEM images of vesicles formed by lipid **11**. e) Phase contrast (left) and fluorescent images (right) of the vesicles formed from lipid **14**. f) Photoligation reactions between lysosphingomyelin **17** and alkyl substituted 2-nitrobenzyl aldehyde derivatives **16**. g) Microscopy images of the dynamic generation of lipid vesicles from **16/17** aggregates triggered by light. h) TEM images of the vesicles; the one in down is negatively stained by phosphotungstic acid. i) Imine formation reaction between the aldehyde precursor **19** and dodecyl amine **20** to form lipid product **21**. j) Phase contrast micrographs of **21** before and after light pulse. Figures adapted with permission from (a), (c), (d) reference^[52]. Copyright 2018 American Chemical Society; (b), (e) reference^[53]. Copyright 2022 Wiley-VCH GmbH; (f)-(h) reference^[55]. Copyright 2021 Royal Society of Chemistry; (i)-(j) reference^[56]. Copyright 2023 American Chemical Society.

Light thus acted not only as a trigger for lipid formation but also as a regulator of membrane stability, enabling spatiotemporal control over compartmentalisation. Such photoresponsive approaches expand the toolkit for constructing synthetic compartments by allowing spatial and temporal control over membrane assembly.

1.4 Out-of-Equilibrium (OOE) Vesicles

Although the above-mentioned strategies have enabled excellent progress in the in situ generation of phospholipids^[57] and vesicles for diverse functions, it is important to recognize that equilibrium vesicles remain inherently limited by their static nature. The conventional vesicles assembled at equilibrium have been invaluable as model compartments^[58], but they lack the dynamic remodeling and responsiveness that characterize biological membranes. This makes them a static mimic rather than functional analogues of living systems. In contrast, biological membranes exist under continuous nonequilibrium flux, where lipid synthesis and degradation processes dynamically regulate membrane composition and function.^[59] This contrast provides the motivation to move beyond equilibrium assemblies toward OOE vesicle systems that capture the transient and dissipative character of living membranes.

Systems Chemistry is still in its infancy when it comes to designing active vesicles with life-like traits, where structures that consume chemical energy and display life-like dynamics beyond what equilibrium assemblies can offer. So far, only a handful of studies have demonstrated vesicles that function under truly active, OOE conditions. Most of these examples rely on non-phospholipid building blocks such as surfactants, thioalkanes, or peptide-based amphiphiles rather than canonical phospholipids.

1.4.1 Non-Phospholipid Vesicles

One of the earliest examples of fuel-driven vesicles was reported by Prins and co-workers^[60], who showed that vesicles could be assembled transiently through the consumption of a fuel, effectively turning the fuel into a structural cue (Figure 1.6a). They used a surfactant **22**, which contains a zinc-coordinated triazacyclononane headgroup with a positive charge and a hydrophobic tail with 16 carbons (Figure 1.6b). At higher concentrations the amphiphile formed micelles, but the addition of adenosine 5'-triphosphate (ATP) significantly reduced the critical aggregation concentration, leading to the formation of stable vesicles with diameters of 50–100 nm (Figure 1.6c-e). The vesicles were stabilized by strong interactions between ATP and the **22**, preventing spontaneous disassembly. The addition of an enzyme, potato apyrase hydrolysed ATP into adenosine 5'-monophosphate (AMP) and inorganic phosphate. Since none of these products can stabilize the bilayer, the vesicles disassembled, thereby completing a transient assembly–disassembly cycle with

a well-defined lifetime (Figure 1.6f). Importantly, this process could be repeated multiple times simply by adding fresh ATP fuel.

Beyond their dynamic behaviour, these vesicles also acted as catalytic compartments. Their hydrophobic bilayer served as a microenvironment for reactions that proceed inefficiently in bulk water. The presence of vesicles significantly increased the reaction yield of a nucleophilic substitution between 4-chloro-7-nitrobenzofurazan and 1-octanethiol and importantly, higher ATP concentrations correlated with even greater product formation. This study provided a clear proof-of-principle that vesicles need not be static equilibrium structures but can be engineered to operate under OOE conditions, with their stability, function and lifetimes tied directly to the turnover of the chemical energy source.

Advancing this concept, Prins and co-workers also demonstrated that the addition of a substrate can induce the formation of vesicular assemblies which in turn act as co-operative catalyst for the hydrolysis of the same substrate.^[61] In their system, the assembly process itself created catalytic hotspots without the need for external enzymes, thereby linking the dynamic behaviour of vesicles with substrate-driven reactivity. To explore whether this substrate-induced templating and catalysis can be combined within a single OOE system, they focused on phosphodiester hydrolysis, using 2-hydroxypropyl p-nitrophenyl phosphate (HPNPP) as a model substrate. Owing to its negatively charged phosphate group, HPNPP binds strongly to the headgroup of the amphiphile **22**. This interaction allowed the substrate to play a dual role of inducing the self-assembly of the amphiphiles into vesicles and simultaneously generating the catalytic environment required for its own breakdown. This study was significant because it illustrated a general principle where substrates can help organize their own catalytic machinery through OOE self-assembly. Such a feedback mechanism moves beyond the idea of vesicles as passive containers and shows how active supramolecular systems can integrate structure, dynamics, and function. For the broader field, these works provided a compelling blueprint for how OOE assemblies might achieve increasingly life-like behaviour, by coupling energy consumption, structural adaptation, and chemical reactivity in a single framework.

Following the idea of coordination driven transient vesicles, Rana's group introduced *Vesi-Glue*^[62] and *Vesi-NTA*^[63], which extended the self-assembly to more complex vesicular architectures (Figure 1.6g-i). In *Vesi-Glue*, guanidinium-bearing surfactants **23** and ATP co-assembled into bilayer vesicles through multivalent non-covalent interactions (Figure 1.6g). ATP again played a central role, by not only stabilizing the guanidinium-based surfactant vesicles but also presenting functional groups for the non-covalent attachment of enzymes to the vesicle surface via salt bridges, hydrogen bonding, and aromatic interactions (Figure 1.6h). The vesicle membrane created a dynamic catalytic platform that enhanced the reaction efficiency of attached enzymes by increasing local

concentrations of enzymes and substrates. As a result, significant catalytic amplification was observed for individual enzymes such as cytochrome C (CytC), horseradish peroxidase (HRP), and laccase, as well as for multistep cascade reactions such as glucose oxidase (GOx)/CytC. The addition of potato apyrase hydrolyses ATP, thereby destabilizing the vesicles and driving the system OOE. This allows catalytic activity to be switched on and off by modulating ATP concentration, providing reversible control reminiscent of temporal regulation in biological systems. Despite these advantages, the enzymatic byproducts compete with ATP in binding, gradually reducing the efficiency of vesicle reformation and attenuating catalytic performance over multiple cycles.

The concept was further expanded in the Vesi-NTA system, where the energy input shifted from nucleotides to metal-ion templating. The system was based on a three-component coordination motif containing an imidazole-containing amphiphile **24**, Cu (II) ions, and a tricarboxylate chelator **25**. Cu (II) ions act as dynamic glue, bridging two amphiphiles with **26** in a cis-orientation to generate lipid-like constructs **31**, that eventually self-assembled into bilayer vesicles (Figure 1.6i). These metallo-lipoid vesicles displayed remarkable catalytic versatility. Within their hydrophobic bilayers, they accelerated nucleophilic substitution reactions (S_NAr), achieving an increase in the rate compared to bulk solution, with activity tuned by substrate hydrophobicity and chain length. At the same time, by introducing functional groups such as primary amines, the vesicles supported aqueous covalent catalysis, dramatically enhancing reactions such as retro-aldol cleavage (~10-fold increase) and imine bond formation (up to ~350-fold acceleration). Importantly, the assembly and catalytic activity of the vesicles could be oscillated in time by pulsing with Cu (II) and competing chelators like EDTA, offering temporal control of both vesicle

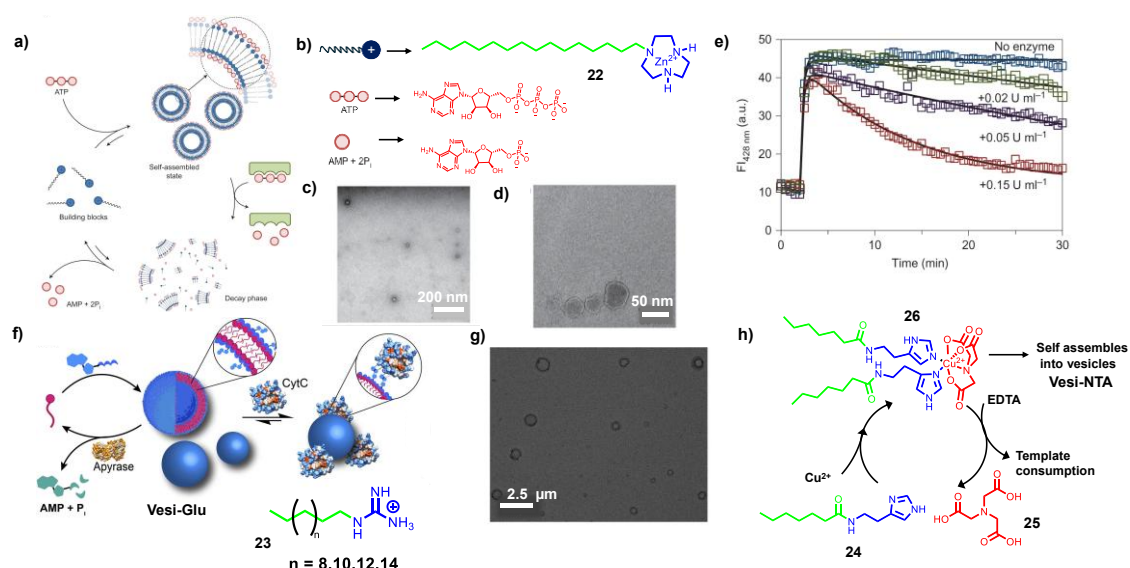


Figure 1.6. a) Schematics showing the assembly and disassembly of dissipative nanoreactor. b) Chemical structures of the surfactant **22**, ATP and AMP. c) TEM image d) Cryo-TEM image of the vesicles formed from **22** and ATP. e) Fluorescent intensity (FI) of DPH at 428 nm as a function of time following addition of ATP to **22** in the presence of different concentrations of potato apyrase.

f) Schematics representation of the transient assembly of Vesi-Glu system showing non-covalently attached proteins and chemical structure of Vesi-Glu system. g) TEM image of Vesi-Glu vesicles. h) Schematic showing the transient assembly of Vesi-NTA driven by Cu^{2+} . Figures reproduced with permission from (a)-(e) reference^[60]. Copyright 2015 Springer Nature; (f)-(g) reference^[62]. Copyright 2023 Royal Society of Chemistry; (h) reference^[63]. Copyright 2024 Wiley-VCH GmbH.

lifetime and reaction output. More broadly, this work demonstrates how metal ion coordination can be harnessed as a OOE control element to achieve compartmentalization, self-organization, and adaptive chemical function, the traits essential for bridging synthetic assemblies with the complexity of living systems.

In a recent study, Lee and co-workers^[64] reported a substrate-induced transient vesicle system in which the components of an aromatic substitution reaction triggered assembly, catalysis, and eventual disassembly. The design was based on a pyridine-based amphiphile with oligoether chains grafted at both the aromatic ends, which self-assembled into vesicular nanostructures of roughly 200 nm in diameter upon the addition of a hydrophobic thiol and an aromatic halide. Dynamic light scattering (DLS) confirmed the emergence of vesicular aggregates under these conditions. Within the vesicular state, the close proximity of the substrates facilitated the $\text{S}_{\text{N}}\text{Ar}$ reaction. Control experiments confirmed that both the spatial confinement and the pyridine microenvironment contributed to the enhanced catalytic effect. As the reaction proceeds, the HCl generated in situ protonates the pyridine units of the amphiphile, weakening hydrophobic interactions and increasing electrostatic repulsion. This destabilizes the vesicles, causing them to disassemble leading to the release of the hydrophobic product. Notably, the system could be regenerated through the neutralization of the protonated pyridine amphiphile with K_2CO_3 , allowing multiple cycles of vesicle formation, catalytic turnover, and disassembly. By coupling catalysis to a fuel-consuming process, the study provides a conceptual blueprint for designing nanoreactors that integrate acceleration, regulation, and product release into a single cycle.

Although examples of synthetic compartments that truly grow and divide under OOE conditions remain rare, the pioneering work of Boekhoven and co-workers represents an important step forward.^[65] They reported an OOE system capable of generating dynamic synthetic vesicles that mimic the remodeling capacity of biological membranes (Figure 1.7a). Traditional synthetic membranes are typically static, but in this system, a chemical reaction cycle was introduced to drive the continuous exchange and activation of building blocks, enabling life-like adaptability. They used tripeptide precursors (**27** and **28**) bearing two carboxylate groups, which alone cannot self-assemble due to electrostatic repulsion. Upon addition of the carbodiimide fuel **29**, these groups undergo an activation step to form a cyclic anhydride intermediate **30**, which spontaneously hydrolyses back to the precursor. This coupling of assembly to a fuel-driven activation–deactivation cycle generates vesicle membranes composed of a dynamic mixture of the anionic precursor and neutral anhydride product (Figure 1.7b). Remarkably, the authors also demonstrated that this OOE

process could be pushed beyond simple assembly–disassembly cycles to generate vesicle transformation and division. Experimentally, the system exhibited striking transient behaviour after fuel addition, vesicles appeared within minutes, initially as small spherical assemblies ($\sim 0.5\ \mu\text{m}$), then evolving into larger multilamellar structures hundreds of nanometres to several micrometres in size. Remodeling was observed throughout their lifetime, with membranes transitioning from rugged to smoother surfaces before eventual decay. Kinetic analyses confirmed that activation and deactivation processes were continuously operating during assembly, while NMR spectroscopy and simulations revealed the importance of the mixed precursor/product composition for stability. This behaviour mirrors some of the hallmarks of protocells, highlighting how nonequilibrium fueling can endow synthetic vesicles with dynamics reminiscent of growth, remodeling, and division in living systems.

Extending this approach, the same group later employed a similar strategy to drive the formation of fatty acid vesicles.^[66] These fatty acid vesicles exhibited highly dynamic behaviour, undergoing budding and fission into smaller daughter vesicles while retaining encapsulated cargo, a key feature associated with protocell-like function. However, the division process was not uniform; the events were heterogeneous, producing daughter vesicles of varying sizes and orientations, including both inward- and outward-budding. Overall, these works establish a minimal, fuel-driven route to vesicle division, bridging the gap between static membrane models and dynamic protocell behaviour. While still primitive compared to biology, it represents a crucial conceptual advance toward building synthetic cells capable of autonomous replication and helps illuminate how simple chemical processes might have supported the earliest forms of cellular life.

Like division, autopoiesis is also considered as a defining characteristic of living systems where a compartment sustain itself through continuous processes of growth, transformation, and eventual decay, driven by internal reaction networks and exchanges with the environment. In biological context, such self-maintenance ensures that cells not only preserve their boundaries but also dynamically remodel them in response to energy fluxes. Very recently Das and co-workers^[67] reported an elegant approach to OOE vesicles through the spontaneous generation of autopoietic compartments from simple bolaamphiphile building blocks (Figure 1.7c). In this system, a dipeptide **31** and an activated amphiphilic ester **32** reacted reversibly to form **33**, that self-assembled into vesicles. These vesicles were not passive containers but displayed intrinsic hydrolase-like activity, catalysing the cleavage of their own building blocks (**34** and **35**). This self-degradation represented a negative feedback loop that eventually led to disassembly (Figure 1.7d). At the same time, the reaction releases protons, lowering the pH and enabling the regeneration of **31** from a precursor **36**. This positive feedback extended vesicle lifetimes, creating a minimal reaction network that sustained the vesicles under OOE conditions. The key novelty of this work was the successful integration of both negative and positive feedback loops, giving rise to autonomous assembly and

disassembly cycles with minimal building blocks, thereby moving a step closer to mimicking the autopoietic behaviour of living cells. Such studies provide valuable insights into how protocell-like systems might have sustained themselves in the absence of enzymes. Nevertheless, the study remained restricted to demonstrating the transient formation and breakdown of vesicles driven by intrinsic catalytic activity as a single cycle.

While such systems capture the self-maintaining features of living compartments, it is equally important to recognize the underlying molecular processes that drive and regulate this behaviour. One of the most fundamental among these is autocatalysis, where the products of a reaction accelerate their own formation. Autocatalytic reaction networks not only provide a chemical basis for sustained vesicle growth but also introduce nonlinear dynamics, feedback regulation, and the potential for evolutionary selection.^[68] In this context, Fletcher's group has employed autocatalytic thiol–disulfide chemistry to construct self-replicating amphiphiles capable of sustaining themselves under OOE conditions.^[69] Expanding this, the authors replaced the linear surfactant with a double-tailed analogue to capture features of cellular membranes within an OOE metabolic cycle. This subtle yet powerful design change promoted bilayer organization, enabling the emergence of stable vesicular assemblies. Using phase-separated precursors (**37** and **38**), the authors achieved the autocatalytic formation of a branched, double-tailed surfactant **39** that spontaneously self-assembled into vesicles (Figure 1.7e). Small micelle-like structures first emerged, which subsequently matured into ~150 nm vesicles (figure 1.7f). Remarkably, these vesicles exhibited autopoietic behavior, sustaining their own existence through the continuous fuel-driven cycle and representing one of the earliest examples of chemically fueled, OOE vesicle replicator. By introducing **40** as a nucleophilic catalyst, the breakdown of the surfactant was accelerated (**41**), establishing a second catalytic cycle alongside the autocatalytic formation process (**42**). This modification allowed the system to be continuously powered by hydrogen peroxide, which regenerated the disulfide precursor (**43**) and completed the metabolic network (Figure 1.7g). The interplay of autocatalytic growth, catalyst-driven breakdown, and continuous fueling sustained the vesicles in a dynamic steady state, with disassembly occurring only when the fuel supply was halted. Conceptually, this work is important because it demonstrates how coupled catalytic cycles can be used to sustain self-replicating vesicles under OOE conditions. The study also strengthens the connection to prebiotic models, where primitive compartments capable of growth, turnover, and persistence would have been essential for the emergence of protocells.

Furthermore, the same group developed a chemically fuelled vesicular oscillator that mimicked time-controlled behaviours similar to biological rhythms, such as circadian rhythms.^[70] The system relied autocatalytic formation as well as the autocatalytic decomposition of the surfactant leading to the formation and collapse of vesicle populations (Figure 1.7h). This deliberate integration of positive and negative feedback within a dissipative chemical network provides a blueprint for

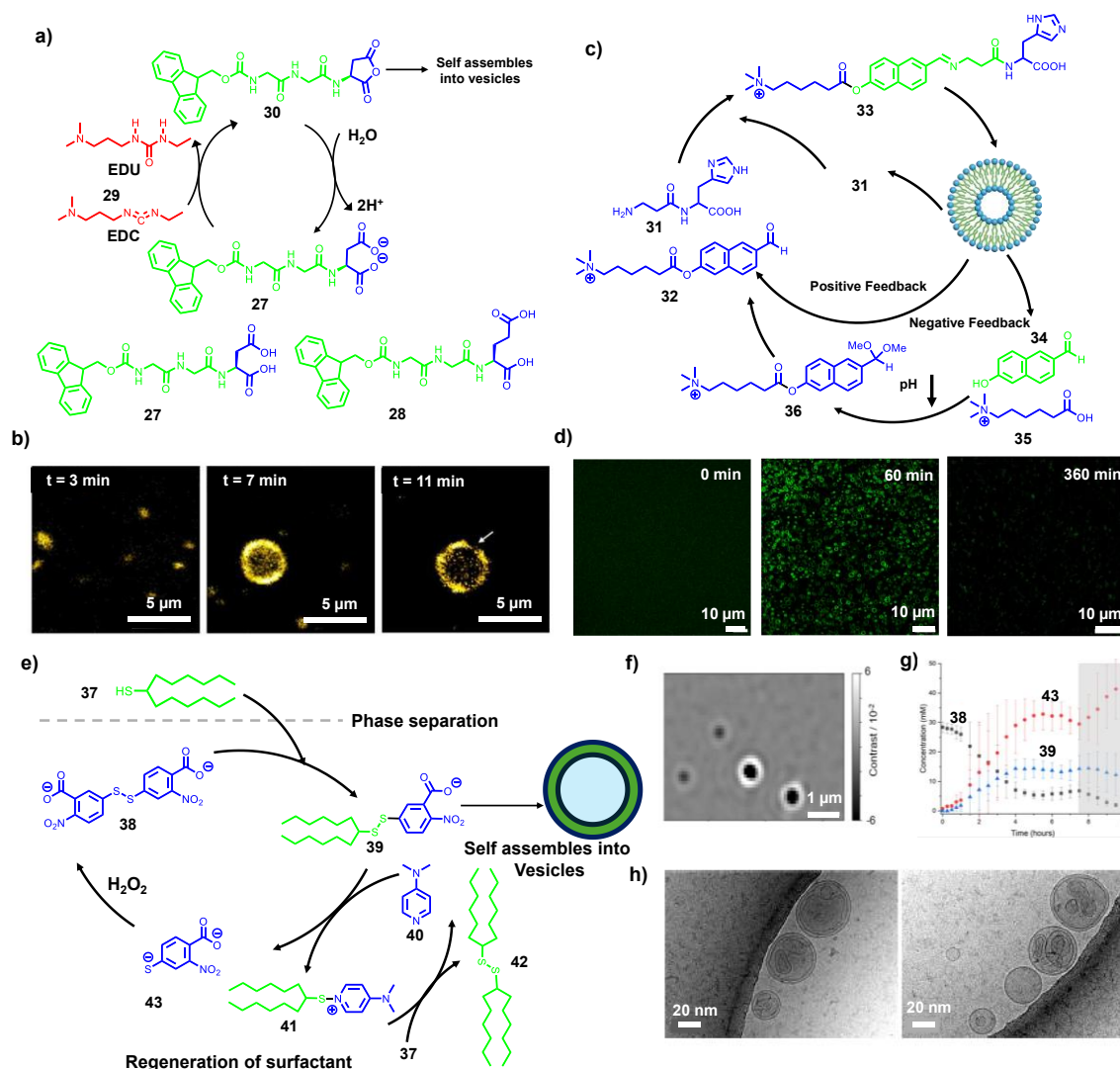


Figure 1.7. Reaction cycle showing the formation of transient anhydride **30**. **b)** Time dependent confocal images showing the formation and disintegration of vesicles formed using Nile red. **c)** Chemical cycle showing the feedback driven formation of **33**. **d)** Time-dependent confocal microscopy images of vesicles in the presence of fluorescein dye. **e)** Chemical reaction cycle for the OOE formation of autopoietic vesicles composed of surfactant **39**. **f)** Interferometric scattering microscopy (iSCAT) image self-assembled **39**. **g)** Continuous hydrogen peroxide fueling sustains the autopoietic system OOE, while discontinuation (grey region) results in the thermodynamic equilibrium. Figures adapted with permission from (a)-(b) reference^[65]. Copyright 2019 Wiley-VCH GmbH; (c)-(d) reference^[67]. Copyright 2025 Elsevier; (e)-(h) reference^[69]. Copyright 2020 Wiley-VCH GmbH.

engineering rhythmic behaviours into synthetic compartments, bridging molecular reactivity with life-like dynamics.

Light-driven oscillations in vesicle-forming systems have also been demonstrated by Pérez-Mercader and co-workers.^[71] In their approach, light-triggered radical polymerization produced giant polymer vesicles that displayed striking life-like behaviours, including cyclical growth, collapse and regrowth as well as directed movement in the form of phototaxis.

Most of the examples discussed above focus either on OOE strategies designed to enhance catalytic activity under dissipative conditions or on autopoietic vesicles that undergo continuous cycles of assembly and disassembly. A particularly significant advance was reported by Zhao et al.^[72], who introduced a membrane-inspired OOE assembly system capable of switching between distinct biological functions using chiral supramolecular building blocks. They used a benzoyl cysteine derivative, that self-assembled into stable M-helical nanofibers under equilibrium conditions. These equilibrium structures formed a bioactive M-helix nanofiber that promoted cell proliferation via stereospecific interactions with chiral cell membranes, while also serving as a reservoir for hydrophobic drugs such as doxorubicin. Upon the addition of a chemical fuel, N-methylpiperazinothiuram disulfide, the disulfide–disulfide metathesis reaction induced a transient structural reorganization of the assemblies. The helical nanofibers are structurally reorganized into positively charged hollow nanospheres. In this OOE state, the nanospheres enabled rapid release of the drug, promoted cellular uptake by cancer cells, and facilitated lysosomal escape, ultimately leading to effective cell death. Once the fuel is consumed, the nanospheres relax back to the original M-helix nanofiber state, enabling recycling of the encapsulated drug and restoring the fibroblast-promoting function. This fuel-gated switching between equilibrium nanofibers and transient nanospheres offers programmable control over payload. By integrating biocompatible supramolecular design with energy dissipation, this study illustrates how minimal molecular components can be programmed to deliver complex, adaptive behaviours, bridging the gap between the OOE concepts and functional biological applications.

Although the creation of robust bilayer structures from simple building blocks under OOE conditions remains a challenge, these examples collectively illustrate how compartmentalization can be regulated through chemical inputs and coupling it with growth, division, and decay, the features central to both living matter and the design of adaptive synthetic systems.

1.4.2 Phospholipid Vesicles

While synthetic approaches have commonly relied on the assembly of pre-formed phospholipids to construct protocell models or drug delivery vehicles, biological membranes are in fact generated in situ by anabolic processes that consume chemical energy such as ATP and degraded through catabolic pathways mediated by enzymes such as phospholipases.^[59, 73] For instance, cell membranes dynamically adjust their lipid composition through the Lands' cycle, a process involving repeated deacylation and reacylation of phospholipids. In this pathway, phospholipase A₂ removes a fatty acid from the sn-2 position of a phospholipid to produce a lysophospholipid, which is then reacylated by specific acyltransferases.^[74] This bidirectional flux underlies the adaptive and time-dependent character of cellular membranes. Introducing synthetic reaction networks capable of regulating such activation and deactivation processes could allow the fabrication of more

advanced, compartmentalized systems with emergent properties such as spatiotemporal control and gradient formation.

Although numerous non-phospholipid systems have been developed to mimic life-like behaviors, recreating the metabolic activity inherent to natural cell membranes remained a central challenge. Addressing this, Post et al.^[75] developed self-reproducing phospholipid vesicles using a Cu-catalyzed alkyne-azide cycloaddition (CuAAC) reaction, in which the newly formed vesicles acted as phase-transfer catalysts, accelerating their own growth (Figure 1.8a). Their system utilized the CuAAC reaction between a hydrophilic phosphocholine **44** (aqueous phase) and a lipidated azide **45** (organic phase) to generate phospholipid **46**. Once the concentration exceeded the critical aggregation concentration (CAC), **46** assembled into vesicles. These vesicles encapsulated the hydrophobic tail precursor, positioning it in close proximity to the aqueous head group precursor and thereby accelerating production of **46**. OOE self-assembly was maintained by coupling vesicle formation with a hydrolysis step that continuously degraded the surfactant, ensuring that the system operated away from equilibrium, much like natural cells that consume energy to preserve function. The model also revealed rudimentary forms of competition and selection where vesicles and micelles derived from different precursors competed for the same feedstock, and the outcome depended on pH. Vesicles prevailed under basic conditions due to their enhanced hydrolytic stability, whereas micelles dominated under neutral conditions by sequestering both reactants and the Cu catalyst, effectively blocking vesicle growth. Beyond replication and competition, the protocells also supported orthogonal reactivity. By embedding a proline-derived organocatalyst into their membranes, the vesicles catalyzed a secondary aldol reaction, functioning as enantioselective nanoreactors that couple self-reproduction with metabolic-like activity. Although this study provided an elegant demonstration of an OOE lipid system, the biphasic setup and the requirement for copper catalysis restrict the immediate applicability of this strategy in biologically relevant contexts. Also, when compared to other OOE system, this system cannot regenerate the precursors once it is hydrolysed.

In another study, Sutherland and co-workers^[76] demonstrated that simple, energy-driven chemical cycles could provide a plausible route to the emergence of stable membranes on the early Earth. Using non-enzymatic acylation of glycerol-2-phosphate with activated fatty acids (N-acylimidazolides) of varying chain lengths, they generated a library of diacylglyceride products. Hydrolysis of these esters completed the energy dissipative cycle, with shorter-chain species breaking down more rapidly than their longer-chain counterparts. Through repeated acylation–hydrolysis cycles, the system became enriched in longer-chain amphiphiles, which formed stable vesicles. Although this work elegantly illustrates how repeated energy dissipation can drive the selective accumulation of self-assembling amphiphiles, major limitations remain when viewed in the broader context of protocell research. In a related approach, Boekhoven and co-workers^[77]

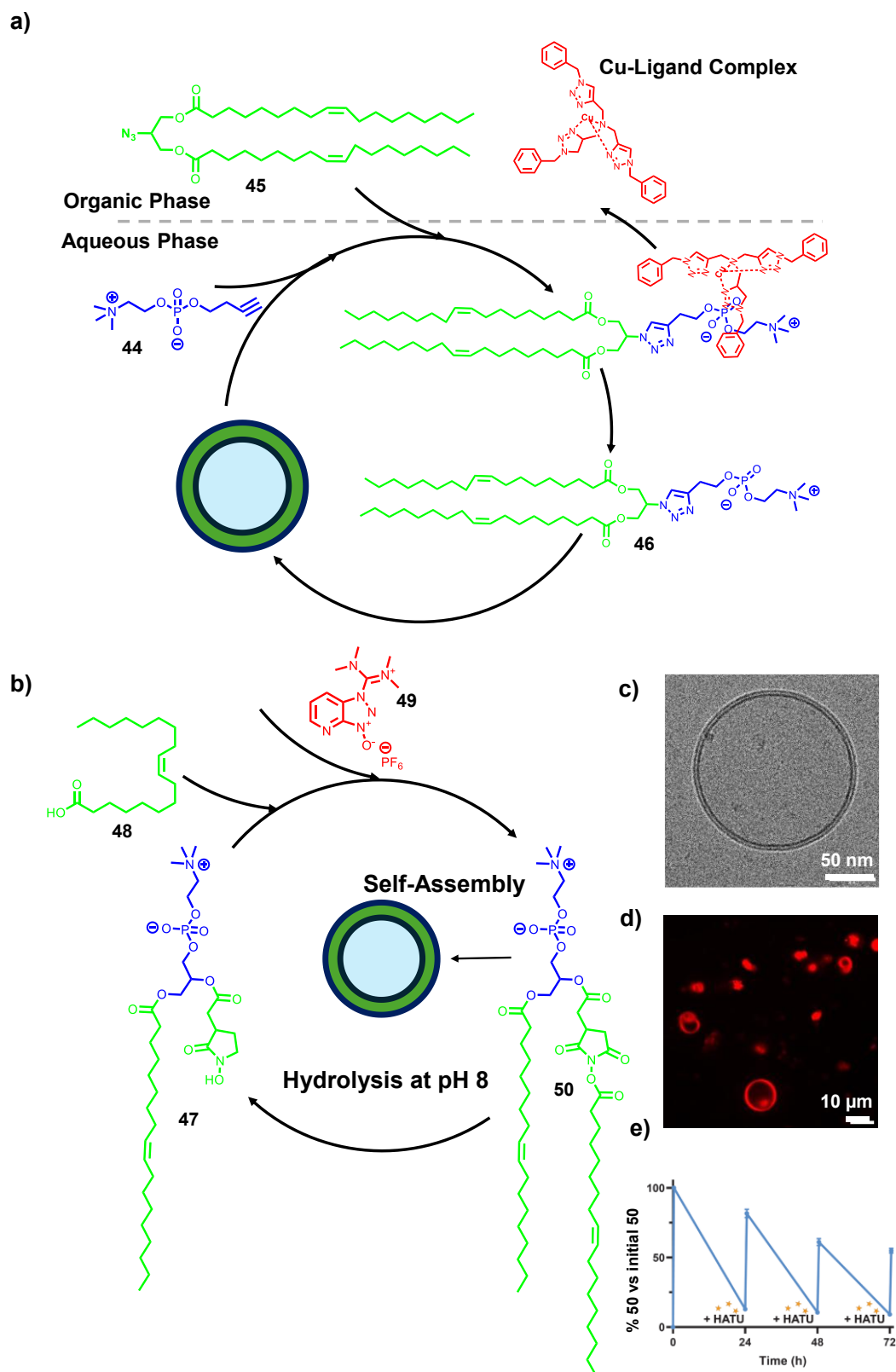


Figure 1.8. a) Reaction scheme of self-reproducing vesicles. b) Reaction scheme for the synthesis of phospholipid **50** by coupling agent **49**. c) Cryo-TEM image of vesicles formed. d) Fluorescence microscopy image of vesicles using 0.01 mol% Texas Red-DHPE. e) Consecutive rounds of formation and hydrolysis of phospholipid by addition of **49** in each cycle. Figures adapted with permission from (a) reference [75]. Copyright 2020 Royal Society of Chemistry; (b)-(e) [78].

reported the formation of OOE vesicles from prebiotically relevant acyl phosphates. By combining fatty acids and alkyl phosphates in the presence of activating agents such as EDC produced double tailed amphiphiles, that self-assembled into vesicles. These vesicles were short-lived and readily hydrolysed back to their precursors. In this way, compartment formation was directly coupled to chemical fueling, underscoring how transient activation can be harnessed to generate life-like, dissipative phospholipid systems.

Very recently, Devaraj and co-workers^[79] reported a significant study on the formation of dynamic artificial membranes (Figure 1.8b). They utilized an abiotic reaction cycle that continuously builds and breaks down phospholipids, thereby maintaining vesicle dynamics without any enzymatic machinery. In this system, the in situ ligation of lysophospholipids **47** with activated fatty acids **48** fueled by the coupling reagent **49** produced non-canonical phospholipid **50** that spontaneously self-assembled into bilayer membranes (Figure 1.8c-d). The resulting structures were inherently transient where the hydrolysis reverted them to their precursors, generating a dynamic flux of lipid synthesis and degradation that closely resembled the metabolic plasticity of biological membranes (Figure 1.8e). Upon fuel depletion, hydrolysis dominated, leading to membrane disassembly and release of encapsulated cargo, whereas continuous fueling preserved vesicle integrity and enabled the encapsulation of polar fluorescent dyes. Beyond maintaining compartment stability, the system also displayed lipid selectivity, hinting at rudimentary evolutionary processes that may have shaped the emergence of primitive membranes on the early Earth.

This study marks a major step beyond static artificial membranes, demonstrating that even a minimal abiotic reaction network can sustain behaviours that are more commonly associated with living cells. By tuning membrane persistence, biasing lipid composition, regulating phase transitions and enabling lipid mixing, the approach offers new strategies for engineering synthetic compartments with programmable lifetimes and functions. However, the use of HATU as a chemical fuel remain a major limitation. Although effective in driving lipid turnover, HATU is a synthetic, cytotoxic reagent that is neither biocompatible nor biologically relevant, thereby restricting the biomimicry of the system and hindering its translation into cellular or therapeutic contexts.

While the above studies are fascinating and successfully mimic aspects OOE formation of phospholipid and non-phospholipid vesicles, most rely on synthetic building blocks with limited connection to those found in biology. In contrast, natural membranes operate through continuous lipid metabolism, where synthesis and degradation are intricately coupled to cellular energy flux. To move closer to this biological paradigm, it becomes fundamentally important to construct OOE vesicles from phospholipid related building blocks, that not only resemble the chemistry of natural membranes but also provide a platform to explore how dynamic turnover can endow vesicles with

emergent, life-like properties. Beyond their relevance to origins-of-life research, such systems hold promise for drug delivery applications, where transient yet controllable vesicles could encapsulate, protect, and release therapeutic cargo in response to fuel availability or metabolic cues. By bridging systems chemistry with true cellular functions, this approach advances from simple protocell models towards vesicles that not only capture the metabolic essence of natural membranes but also inspire the design of smart, adaptive materials for biomedical use.

1.5 Motivation and Objective of the Thesis

Living systems exist far from equilibrium, continuously consuming chemical energy in the form of ATP and GTP to sustain structures and functions that would otherwise collapse. At the heart of this principle lies the cell membrane, which is not a passive boundary but a metabolically active interface that continuously remodels, traffics cargo, and orchestrates complex reaction networks. This dynamic compartmentalization is central to life in which the membranes not only provide structural integrity but also regulate flux, catalysis, and communication with the environment. Mimicking such life-like behaviours remains one of the central challenges of systems chemistry and supramolecular chemistry.

Traditional synthetic vesicles, although invaluable as models of cellular organization, are typically equilibrium structures. They are stable once formed but lack the ability to adapt, remodel, or couple their existence to energy flux. As a result, they cannot capture the transient, responsive, and functional nature of biological compartments. To bridge this gap, chemists have begun to design active vesicles whose formation, stability, and function are sustained by chemical fuels or OOE processes. OOE supramolecular chemistry offers a way forward, by introducing continuous energy dissipation into vesicular systems, thereby allowing them to undergo growth, remodeling, and programmed function. Despite its fundamental importance, replicating and demonstrating the exact OOE dynamics of cellular membranes in synthetic systems has remained a significant challenge, with no reports that replicate these features in a phospholipid system.

This context motivates the design and study of OOE vesicles that operate through metabolic processes, inspired by the energy-driven dynamics of cellular membranes. Such systems hold promise for bridging synthetic supramolecular chemistry with biology by providing functional compartments capable of adaptive behaviour and potential biomedical applications. By embedding chemical reactivity, catalytic turnover, and feedback regulation into vesicular assemblies, this research aims to move synthetic compartments closer to life-like systems capable of adaptive, transient, and functional behaviours. Achieving this would push systems chemistry beyond minimal self-organization into the realm of biologically inspired, biologically relevant, and potentially biologically integrable systems, essentially creating materials *of biology, by biology, for biology*.

The objective of this thesis is to explore how chemical energy can be harnessed to drive the formation and remodeling of phospholipid vesicles under OOE conditions, with the broader vision of advancing systems chemistry toward life-like, biologically relevant architectures. Living cells continuously regulate their membrane composition through complex metabolic pathways, enabling adaptability, compartmentalization, and selective functionality. Inspired by this plasticity, the present work aims to establish minimal synthetic models in which lipid vesicles are not static assemblies but dynamic structures that form, degrade, and remodel in response to chemical fuels and enzymatic cycles.

The specific objectives are as follows:

1. To synthesize water-soluble precursors capable of undergoing chemical transformations to form phospholipids from simple building blocks.
2. To establish the OOE generation of phospholipids through competing anabolic and catabolic reaction with controllable kinetics.
3. To show that continuous supply of chemical fuel can maintain phospholipid vesicle assemblies under OOE conditions.
4. To investigate the ability of OOE phospholipid vesicles to encapsulate model cargo molecules and study their controlled release, mimicking functional features of natural membranes.
5. To demonstrate the influence of fuel hydrophobicity by employing precursors with varying carbon chain lengths and correlating tail length with vesicle formation kinetics and stability.
6. To study the dynamic remodeling of phospholipid vesicles under enzymatic or fuel-driven turnover, providing a minimal model of adaptive membrane behaviour.

1.6 References

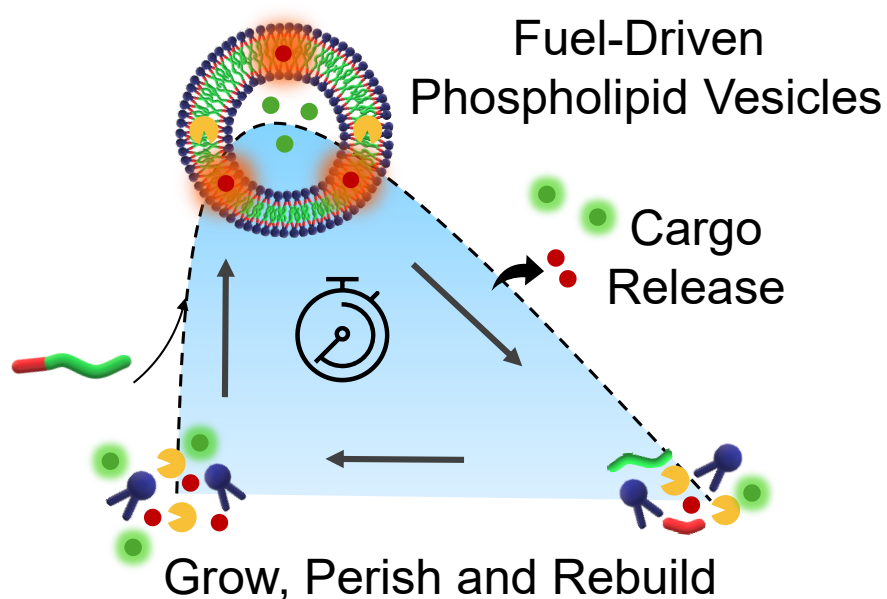
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Chapter 2. Enzyme Controlled Transient Phospholipid Vesicles for Regulated Cargo Release



*This chapter is adopted from the publication: A. Venugopal, S. Ghosh, A. Calò, G. M. Tuvèri, G. Battaglia, M. Kumar, Enzyme Controlled Transient Phospholipid Vesicles for Regulated Cargo Release. *Angew. Chem. Int. Ed.*, **2025**, 64, e202500824.*

2.1 Introduction

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 various transient self-assembled nanomaterials^[1-6] using various fuels for application in enhanced catalysis, self-erasing inks, transient electronics, multiple helical states etc.^[7-15] 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 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 phospholipids have mostly utilized self-assembly of pre-formed phospholipids as protocell models, delivery vehicles etc.^[16-21] In contrast, phospholipids 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 phospholipids, whereas the same phospholipids are catabolically degraded by the membrane-bound enzymes like lipase.^[22] Such metabolic processing of phospholipids 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,^[23-27] composed mostly of synthetic thioalkanes and not phospholipids. Devaraj and co-workers, along with others have reported in situ phospholipids 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.^[28-30] They have recently reported an example of fueled, temporally controlled phospholipid vesicles that demonstrate biological membrane plasticity.^[31-32] 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 phospholipids fueled by chemical energy, to form biomimetic lipid vesicles which can dynamically regulate cargo delivery.

2.2 Results and Discussion

We present an in situ synthesis of biomimetic phospholipid 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 phospholipid (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,^[33-34] which can have side reactions in the biological milieu. We have avoided it by using the design of amino-ester fuel. 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 phospholipid GPC-Imine containing ester unit (Figure 2.1). 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 incorporated cargoes can be dynamically regulated.

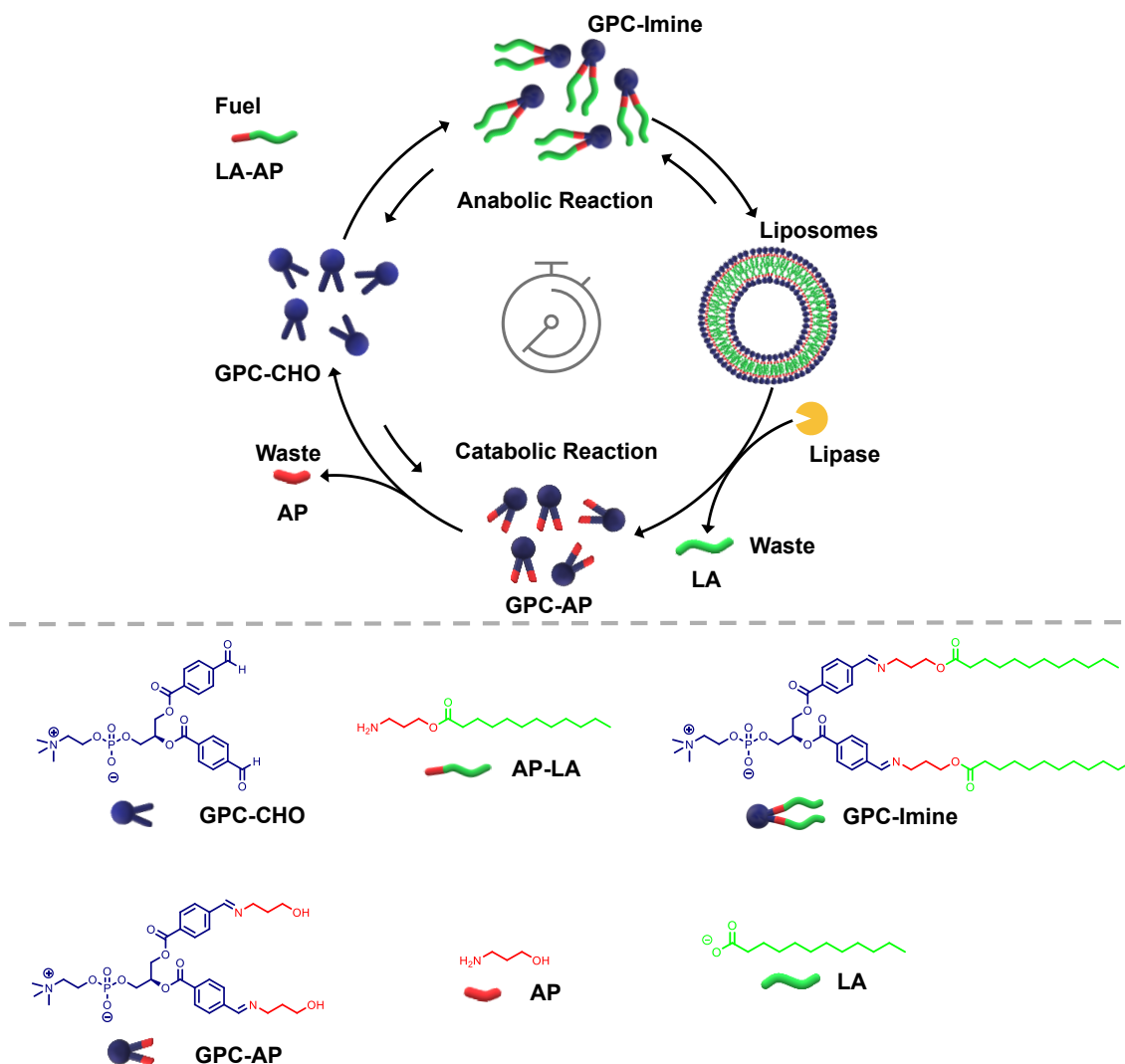


Figure 2.1. Schematic representation of the in situ phospholipid formation and their transient vesicle assembly along with various chemical structures.

Enzymatic reaction assisted transient vesicles have been reported by Maiti et al., however, it is with a completely different chemical design of synthetic surfactant, rather than biomimetic phospholipid.^[35] Thus, we present a novel design of in situ formation of phospholipids, from completely water-soluble precursors, and catabolic degradation of phospholipids, 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 (Figure 2.1) were synthesized as described in Scheme S2.1–S2.2. The chemical design of fuel was to ensure structural resemblance with biological lipids 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 phospholipid vesicles (Figure 2.1).

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 S2.1). 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 2.2a, Figure S2.2–S2.7). 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 2.2b). Thus, NMR and HPLC analysis confirmed an almost complete conversion of GPC-CHO into GPC-Imine.

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 2.2c). 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 2.2d, b).

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 2.2a–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 (Figure 2.2, Figure S2.8).

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 2.3a, Figure S2.9). 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).

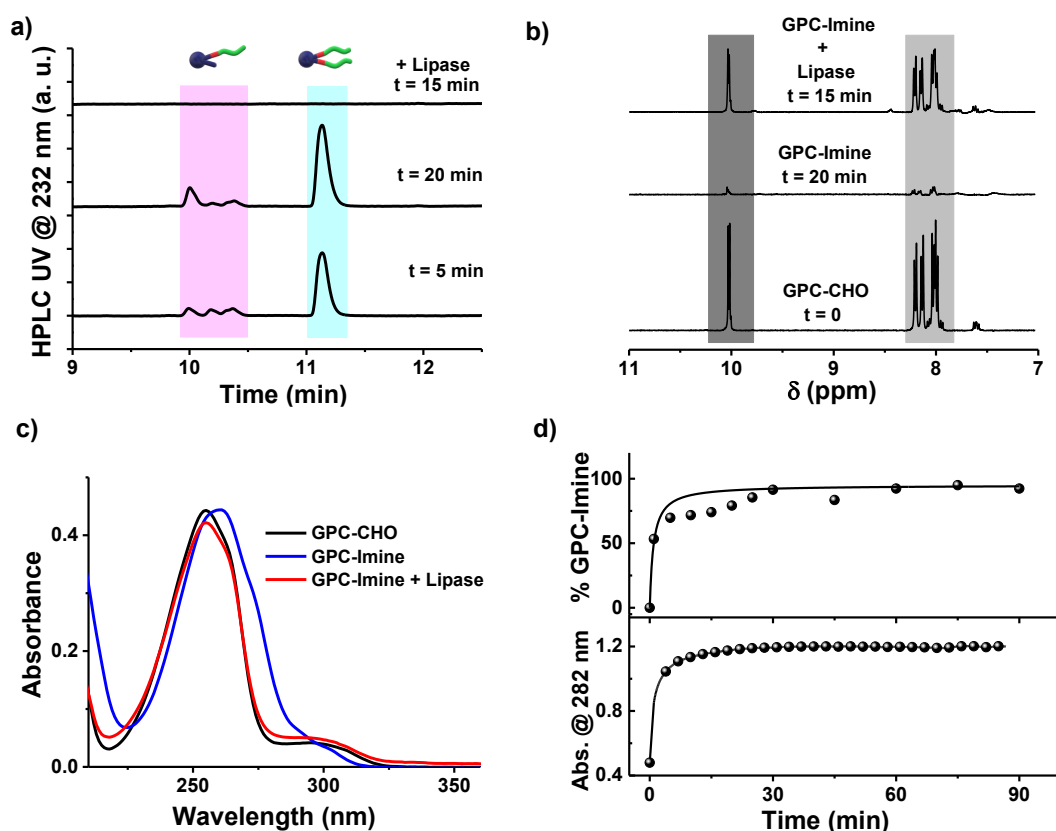


Figure 2.2. a) Time dependent HPLC analysis of GPC-Imine before and after lipase addition. Multiple peaks for single tailed lipid at ≈ 10.19 minutes correspond to chemically different possibilities (Figure S2.4). For HPLC measurement, the GPC-Imine were reduced to their corresponding secondary amine using NaBH_3CN . Thus, HPLC detects only the amine form and not the imine. b) time dependent ^1H 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 $\lambda=282$ nm (bottom, 1 mm cuvette). Conditions: 1 mM GPC-CHO, 3 mM AP-LA, excess lipase, pH 7.4 HEPES buffer.

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. 3.5 μm). These large aggregates were confirmed to be the crystals of lauric acid (LA) waste generated during hydrolysis (Figure S2.10). 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 2.3b–c, Figure S2.11). Moreover, after hydrolysis by lipase, large crystalline structures of waste LA were observed, indicating the complete disintegration of vesicles (Figure 2.3d).

To understand the supramolecular organization, atomic force microscopy (AFM) was used. Lipid vesicles are known to open into bilayer membranes on mica surface.^[36] We observed the formation of ~5 nm thick bilayer membrane (Figure S2.12), similar to the thickness of biological membranes.^[37] The energy minimized structure of GPC-Imine showed a molecular length of ~2.5 nm (Figure S2.13), 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 2.3e). Although confocal is limited to the visualization of large structures, it confirms the structural transformation in solution.

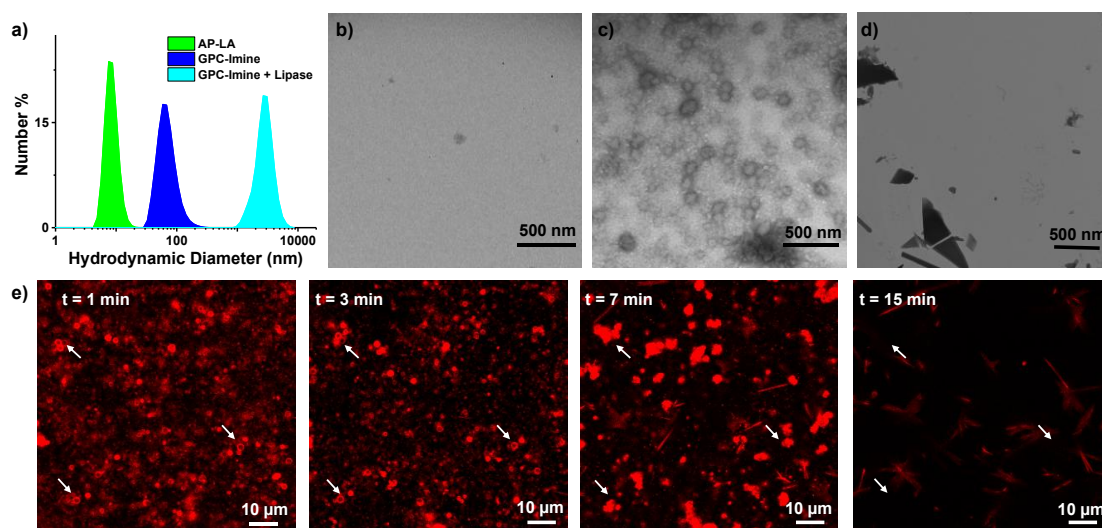


Figure 2.3. a) DLS data showing the change in hydrodynamic diameter when 5 mM GPC-CHO and 15 mM AP-LA react to form GPC-Imine vesicle and upon addition of 600 U/mL lipase (1 hr of incubation after adding AP-LA and lipase). TEM images of b) Fuel AP-LA; c) GPC-Imine vesicles; d) After addition of lipase to the vesicles. e) Snapshots of confocal video imaging of lipase induced disintegration of vesicles with 2000 U/mL lipase. The white arrows highlight a few vesicles that transform over time (over the 4 images). Conditions: 5 mM GPC-CHO and 15 mM AP-LA.

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 2.4a, Figure S2.14–S2.15). 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 S2.16).

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 2.4b, Figure S2.17). However, damping was observed with each cycle, presumably due to the accumulation of waste, which is common in such closed systems. DLS and TEM analysis demonstrated the formation of vesicles upon refueling. However, these formed multilamellar vesicles due to the co-assembly of hydrophobic waste LA (Figure S2.18–S2.22). 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,^[38] it neither reacts with the fuel nor with the GPC-CHO and the selective hydrolysis of the GPC-Imine is facilitated by the formation of vesicle (Figure S2.23). We have also utilized the reactivation capability of our lipid vesicles to temporally control the catalytic hydrolysis of a model hydrophobic ester by lipase (Figure S2.24). Such a reaction is otherwise not possible in aqueous condition and thus highlights the system's potential for transient catalysis.

One of the features of biology is to maintain their OOE structures by continuous metabolism and by constantly consuming fuel. However, synthetic analogs of OOE steady state (NESS) are seldom reported due to their chemical complexity.^[39] Although NESS requires an open system, we aimed to sustain the vesicle by maintaining the concentration of GPC-Imine over time by initially adding an excess of fuel (Figure 2.4c). Thus, we probed the change in the concentration of GPC-Imine using absorbance over time (Figure 2.4d, AP-LA 9 mM). We observed a plateau for up to 75 min, indicating a steady concentration. During this period, 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 controlling the time over which vesicle could be sustained from 5 min to 75 minutes and 105 min to 10 min, respectively (Figure 2.4d–e, Figure S2.25). Thus, we have demonstrated that our chemically fueled vesicles can be sustained over time with easily controllable lifetime (Figure 2.4f). 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 phospholipid GPC-Imine that self-assembles into vesicles of controlled dispersity (PDI=0.221). Additionally, our nanovesicles, with an average size of approx. 65 nm, are optimal for drug delivery applications.

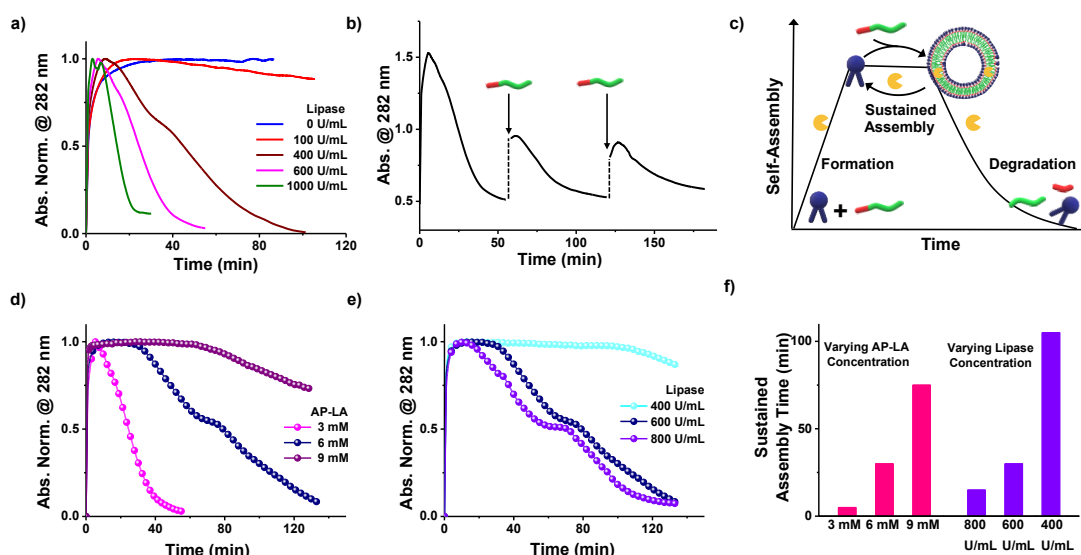


Figure 2.4. a) Time-dependent absorbance changes in presence of lipase showing the autonomous formation and degradation of GPC-Imine resulting in transient lipid vesicles. b) Refueling the system with fresh batch of AP-LA fuel after first cycle with 1 mM GPC-CHO, 3 mM AP-LA, 600 U/mL lipase. c) Schematic showing the need of constant fuel for sustaining GPC-Imine concentration. Absorbance changes showing sustained 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 regulation of time over which GPC-Imine vesicle can be sustained (as 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 S2.26). The data in a), d, e) are normalized between 0 and 1 for clear comparison.

In contrast, traditionally, vesicles formed with phospholipid require complex methods like film rehydration, solvent switch, etc., to result in polydisperse, multilamellar vesicles that need further extrusion procedures to obtain desirable size.^[40] Thus, our vesicle design is highly advantageous which could be due to the in situ formation of the phospholipid 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 localized within the hydrophobic bilayer of the GPC-Imine vesicle by simple mixing (Figure S2.27).

The localization of Nile red on the vesicle membrane was characterized by enhanced fluorescence, and the release was monitored by probing the decrease in fluorescence.^[41] 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 2.5). 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. Furthermore, our in vitro cargo release experiments demonstrated that GPC-Imine vesicles facilitate controlled release of cargo (Nile red) to HeLa cells without significant cytotoxicity (Figure S2.28). Finally, we have also encapsulated hydrophilic dye, pyranine, within the lumen of the GPC-Imine vesicles and examined its release. Our results demonstrate that hydrophilic cargos can also be encapsulated within our vesicles, and their release kinetics can be controlled by varying the concentration of lipase (Figure S2.29). Taken together, these results demonstrate the potential of our system for temporally regulated drug delivery in nanomedicine.

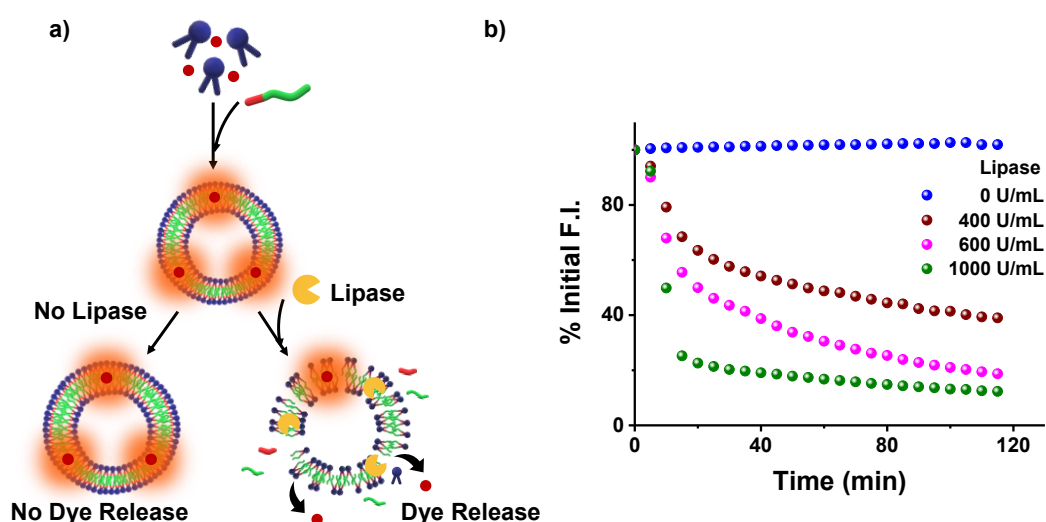


Figure 2.5. a) Schematic showing the localization of hydrophobic cargo, Nile Red, and its release in presence and absence of lipase. b) Release profile of hydrophobic Nile Red from the chemically fueled vesicles. Conditions: 1 mM GPC-CHO, 3 mM AP-LA and 0.25 mM Nile red.

2.3 Conclusion

In summary, we have presented a fuel-driven OOE self-assembly of phospholipid into transient vesicles with a controllable lifetime. In situ formation of phospholipid 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.^[40] These optimally sized vesicles were used to encapsulate model cargoes, and the release kinetics could be dynamically regulated by the enzyme concentration. Thus, our biomimetic design of metabolic phospholipid 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.

2.4. Materials and Methods

2.4.1 Materials

The enantiomerically pure sn-glycero-3-phosphocholine (GPC) was purchased from Biosynth, CymitQuimica. Carboxy benzaldehyde (CBA), and 4-Dimethylamino pyridine (DMAP) were purchased from TCI Europe. Dicyclohexyl Carbodiimide (DCC), 3-(Boc-amino)-1-propanol, Nile Red Dye, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) and Lipase (Candida Rugosa) were purchased from Sigma Aldrich. HEPES sodium salt, Lauryl chloride, n- Nonanoic Acid and Formic Acid were purchased from Fisher Thermos Scientific. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was purchased from Invitrogen. Sodium Cyanoborohydride (NaBH_3CN) and Triethylamine were purchased from Alfa Aesar. Chloroform, Diethyl Ether, Methanol, Anhydrous Dichloromethane (DCM), Cell culture DMSO anhydrous Dimethylformamide (DMF) were purchased from Sigma Aldrich and used without any further purification. All the NMR solvents were purchased from Sigma Aldrich.

Column chromatography was performed on silica gel 60. Analytical TLC was performed on Merck Kieselgel 60 with appropriate mixtures of solvents as eluents. Products were detected by either using Iodine vapors or by spraying the plates with a solution of KMnO_4 followed by heating or 0.05% primuline solution acetone/ H_2O (8: 2, v/v) followed by UV (365 nm) visualization in the case of phospholipid molecules.

2.4.2 Methods

2.4.2.1 NMR Measurements

NMR spectra were recorded with a Bruker AVANCE NEO 400 (400 MHz), Bruker AVANCE NEO (500 MHz) and Varian Mercury (400 MHz) NMR spectrometer with chemical shifts reported in parts per million (ppm). Splitting patterns are designated as s, singlet; d, doublet; bs, broad singlet; m, multiplet; t, triplet; q, quartet; quin, quintet and br, broad.

All NMR experiments for the imine formation, degradation and refuelling experiments were recorded in 100 mM HEPES buffer in D_2O at concentrations of 5mM GPC-CHO and 15 mM AP-LA respectively.

2.4.2.2 UV-Vis Absorption spectroscopy

Electronic absorption spectra were recorded on Shimadzu 1600 UV-Vis Spectrometer using 1 mm and 2 mm quartz cuvettes at a scanning speed of 100 nm per minute from 200-700 nm. For the kinetic experiments the instrument was set to take measurements at an interval of 10 seconds for a period of 2 hours.

2.4.2.3 Transmission Electron Microscopy (TEM)

5 µl of each sample solution was drop-casted onto a continuous carbon-coated grid. The excess solution was removed by blotting the grid with filter paper, followed by two rounds of washing with water and blotting. A negative stain (5 µl of 2% aqueous Uranyl Acetate) was applied and blotted to remove the excess. The samples were dried under air for 2h and then kept under vacuum overnight. TEM Imaging was done using a JEOL 80kv instrument.

2.4.2.4 Confocal Laser Scanning Microscopy (CLSM)

For fluorescent labelling of the vesicles, 0.2 mol percent of Nile red in DMSO was added to the preformed GPC-Imine solution in an Eppendorf tube. The Eppendorf tube was slowly rotated in a rotor for 2-3 hrs at room temperature to facilitate proper incorporation of Nile red into the lipid. After incubation, 10 µL of the sample solution was sandwiched between a glass cover slip and a clean glass slide. The imaging was performed on a Leica TCS SP8 confocal microscope using a 63x/1.40 glycerol-immersion objective and a PMT detector. Images were recorded at 22 °C with a resolution of 1024x1024 pixels and videos at 512x512 pixels at a scanning speed of 400 Hz. Fiji-ImageJ software was used to crop images and adjust contrast and brightness. For the real-time video imaging experiment, we added all the components together and immediately started imaging as mentioned above.

2.4.2.5 High Performance Liquid Chromatography–Mass Spectrometry (HPLC-MS)

The imine formation was quantified using an Agilent HPLC-MS instrument fitted with Poroshell 120 C18 analytical column. A 10 µL of sample at different time points was reduced using an excess of sodium cyanoborohydride (NaBH₃CN, 1mg in ethanol) ^[42] and diluted to 800 µL using acetonitrile: water (50:50) with 0.1% formic acid. This sample was then eluted using Phase A/Phase B gradients (Phase A: H₂O with 0.1% formic acid; Phase B: Acetonitrile with 0.1% formic acid. GPC-Imine formation was confirmed by monitoring the UV signal at 232 nm followed by performing the MS analysis of the same peak.

Injection Volume: 20 µL

Flow rate: 0.400 mL/min

Solvent A: Water + 0.1 % Formic Acid; **Solvent B:** ACN + 0.1 % Formic Acid

Time (min)	Solvent A %	Solvent B %	Flow rate (mL/min)
0	90	10	0.400
2	70	30	0.400
9	10	90	0.400
15	10	90	0.400
16	90	10	0.400
17	90	10	0.400

HRMS data was obtained using the same method in a HPLC-HRMS instrument

2.4.2.6 Reduction of GPC-Imine for HPLC Analysis

Since GPC-Imine, like any imine bond (dynamic covalent bond) is not stable upon dilution, we cannot perform HPLC because HPLC dilutes the sample considerably. Thus, just for HPLC analysis, GPC-Imine was reduced to its more stable secondary amine using NaBH_3CN . In our case, 10 μL of lipid solution was added to a glass vial containing 10 μL of NaBH_3CN (1mg) in ethanol and vortexed for 1 minute to convert GPC-Imine to its corresponding secondary amine. After this the sample was diluted with 800 μL of 50% MeCN in Water (with 0.1% formic acid). Any unreacted NaBH_3CN cannot change the equilibrium towards GPC-Imine formation in HPLC sample as HPLC sample is highly diluted (80 times, 800 μL) which quenches i.e. 1) hydrolyses any leftover GPC-Imine and 2) prevents any further formation of GPC-Imine.

Lifetime analysis: For this work, we have defined lifetime as the time during which there is a detectable concentration of GPC-Imine in the solution.

2.4.2.7 Dynamic Light Scattering (DLS)

DLS measurements were carried out using a Zetasizer Ultra (Malvern UK) employing a 633 nm laser at a back scattering angle of 173° . The number weighted size distribution peak values were used to report the hydrodynamic diameters along with the correlation function curve. Sample volume used for each measurement were 70 μL .

2.4.2.8 Atomic force microscopy (AFM)

AFM images were recorded using a Nanowizard 4 Bio-AFM (JPK-Bruker) with SSS-FMR supersharp cantilevers (Nanosensors) for ambient condition imaging (nominal resonance frequency $f_{\text{res}} = 75 \text{ kHz}$ and spring constant $k = 2.8 \text{ N/m}$). Topography images were collected in both QI mode (data in Fig. S11 a-b) and amplitude modulation AFM (data in Figure S2.12 c-d). Measurements were performed in air at room conditions. 50 μL of GPC-Imine solution was drop cast on freshly cleaved mica and allowed to dry in air for 2 hrs and then kept in a desiccator for 24hrs before imaging. Images in amplitude modulation AFM were collected setting a free amplitude $A_{\text{free}} = 17 \text{ nm}$, and the highest amplitude setpoint compatible with good image quality. QI images were collected after calibrating the cantilever optical sensitivity on sample areas exposing the bare mica and calibrating the cantilever spring constant with the thermal noise tune method. In this case, sample topography was obtained from force curves, by recording the z-piezo movement necessary to reach a fix force setpoint for each image pixel (force setpoint = 5 nN, z-range = 300 nm, pixel time = 9.4 ms, z-speed = 80 $\mu\text{m/s}$).

2.4.2.9 Cell Culture

HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 2 mM GlutaMAX, and penicillin/streptomycin penicillin (100 IU)/streptomycin (100 µg/mL) at 37°C under a humidified 5% CO₂ atmosphere. Subcultures were performed upon reaching confluency, with cells detached using 0.25% (w/v) trypsin/EDTA for 5 minutes at 37°C, followed by centrifugation at 200 RCF for 5 minutes to pellet the cells. Cellular densities were adjusted according to the specific requirements of each experiment. Only cells between passage numbers 7 and 15 were used to ensure consistency across experiments.

2.4.2.10 Cell Viability Assay

HeLa cells were seeded into 96-well plates at a density of 2×10^4 cells per well and incubated overnight at 37°C in a 5% CO₂ atmosphere. After 24 hours, when confluency was achieved, the cells were treated with serial dilutions of stock solutions of GPC-CHO and AP-LA (maximum concentration: 100 µM, prepared in 1× PBS) for 24 and 48 hours. PBS and 20% DMSO served as the positive and negative controls, respectively. Following treatment, the media was removed, and cell viability was evaluated using the MTT reduction colorimetric assay. A total of 100 µL of MTT solution (0.5 mg/mL) was added to each well, and cells were incubated for 2 hours to allow the formation of purple formazan crystals. These crystals were subsequently dissolved in 50 µL of DMSO, and absorbance was measured at 570 nm using a plate reader. Cell viability was calculated as a percentage using the formula:

$$\% \text{ Cell Viability} = (\text{Average Sample Absorbance} / \text{Average Control Absorbance}) \times 100$$

2.4.2.11 Nile Red Uptake Confocal Imaging

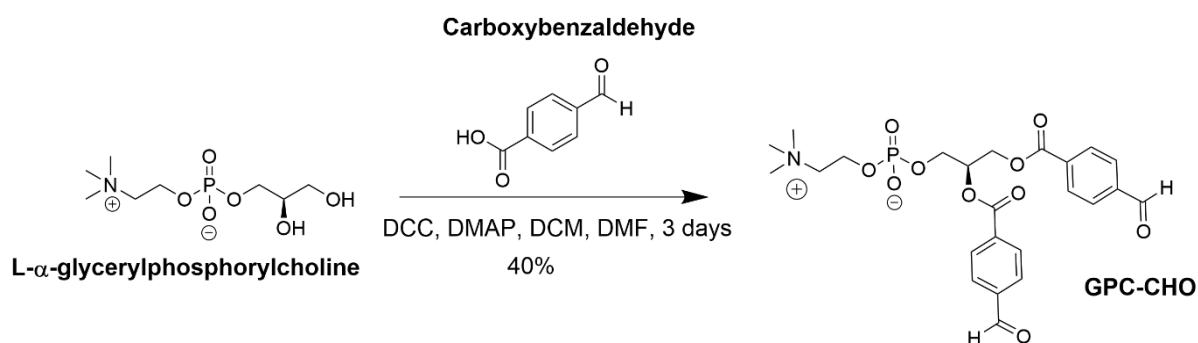
HeLa cells were seeded at a density of 5×10^3 cells per well in a µ-slide 8-well chambered coverslip with ibiTreat surface (ibidi GmbH, Germany) and incubated in 200 µL of culture medium at 37°C with 5% CO₂ for 24 h. Cells were then treated with the following solutions: (a) Nile red only, (b) Nile red containing GPC-Imine, and (c) Nile red containing GPC-Imine and Lipase, and incubated for 30 min at 37°C in 5% CO₂. After incubation, cells were washed with PBS and fixed using 4% paraformaldehyde. Fixed cells were stained with 100 µL of Hoechst 33342 solution (5 µg mL⁻¹ in PBS) for 10–15 min at 37°C to visualize nuclei. Cells were washed twice with PBS, and imaging was performed directly using a Leica SP8 confocal microscope. Images were acquired using a 63× objective lens with the following excitation and emission settings: Hoechst (Ex: 405 nm, Em: 426–472 nm) and Nile red (Ex: 545 nm, Em: 580–680 nm).

2.4.2.12 Nile red Uptake

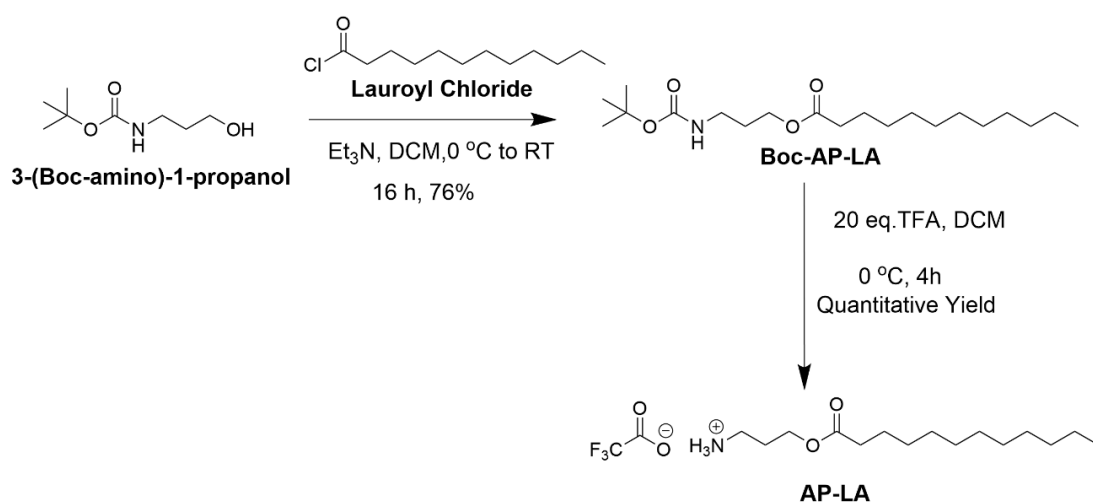
HeLa cells were seeded at a density of 2×10^4 cells per well in a 96-well plate and incubated overnight at 37°C in a 5% CO₂ atmosphere to allow adherence and growth. Upon reaching confluency, cells were treated with the following solutions: (a) Nile red only, (b) Nile red containing GPC-Imine, and (c) Nile red containing GPC-Imine and Lipase, for incubation periods of 15, 30, or 60 minutes. Following treatment, the media was removed, and the intracellular Nile red was extracted by adding 100 µL of DMSO to each well. The fluorescence intensity of the extracted dye was measured using a plate reader with an excitation wavelength of 514 nm and an emission range of 580–680 nm.

2.4.2.13 Synthetic Schemes

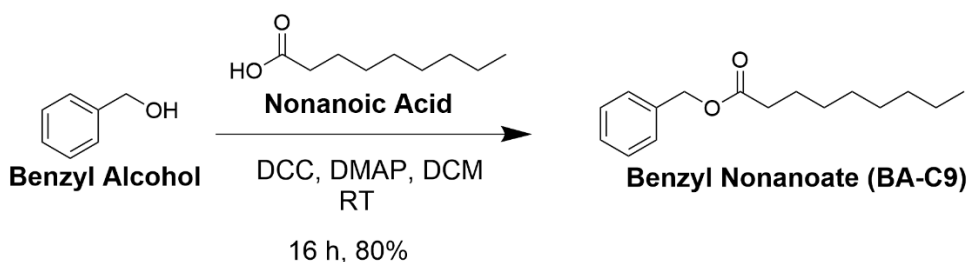
Synthesis of GPC-CHO, AP-LA and BA-C9 are shown in Scheme 1, Scheme 2 and Scheme 3 respectively.



Scheme S2.1: Synthesis of phospholipid head mimic GPC-CHO.



Scheme S2.2: Synthesis of chemical fuel AP-LA.



Scheme S2.3: Synthesis of hydrophobic ester BA-C9.

2.4.2.14 Synthesis of GPC-CHO

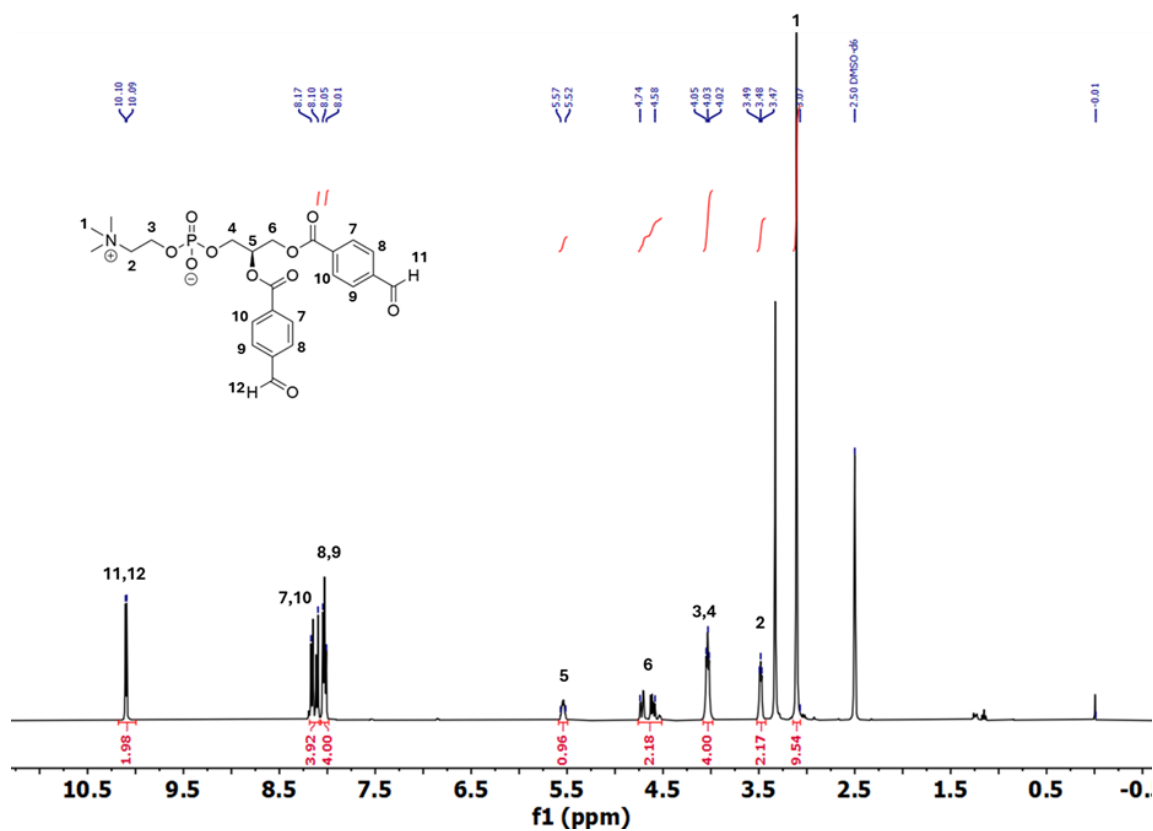
A mixture of L- α -glycerylphosphorylcholine (500 mg, 1.9 mmol), Caboxybenzaldehyde (1.16 g, 7.77 mmol), 4-(N, N-dimethylamino)pyridine (DMAP, 474 mg, 3.8 mmol), and N, N-dicyclohexylcarbodiimide (DCC, 7.77 mmol, 1.6 g, 0.97 mmol) was stirred for 72 h at 40 °C in anhydrous CH_2Cl_2 (30 mL) and DMF (5 mL), under nitrogen atmosphere. The reaction progress was monitored by TLC. When the reaction was completed, the urea precipitate formed during the reaction was filtered off and the solvent was evaporated in vacuo. The crude product was purified using silica gel-based column chromatography with the gradient $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (65: 25: 4, v/v).

Yield: 390 mg, (40%)

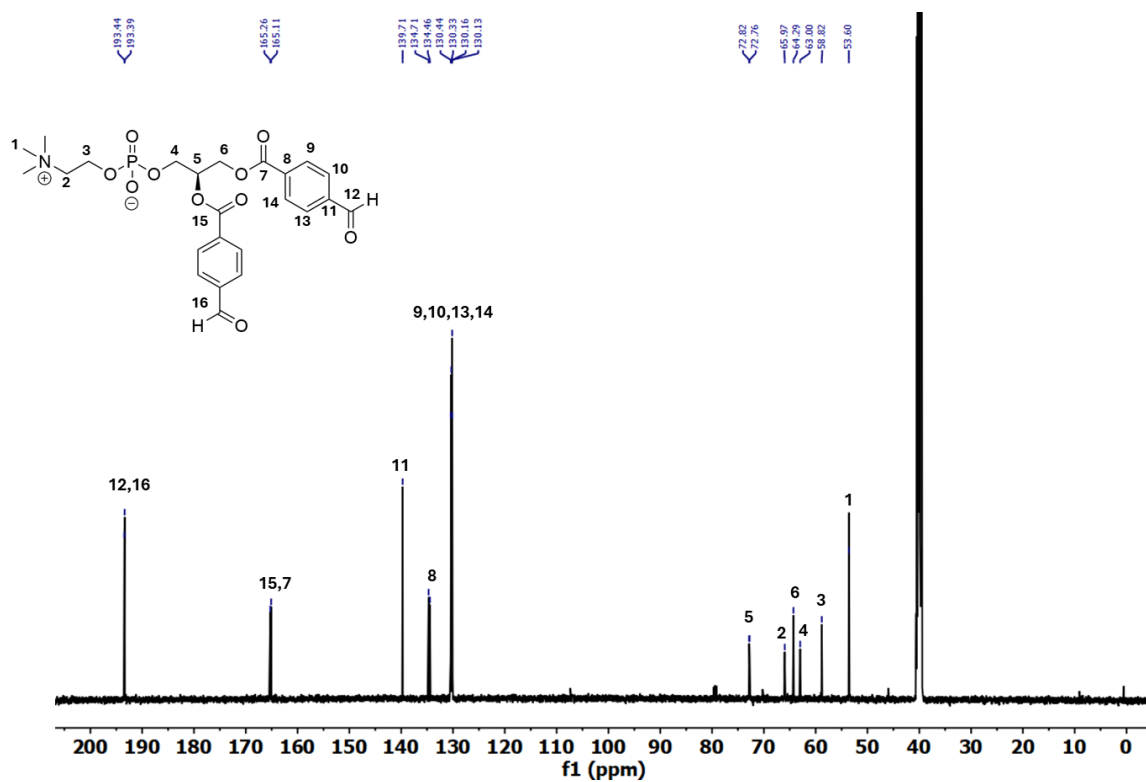
^1H NMR: (400 MHz, DMSO-d_6): δ 10.10 (d, $J = 4.4$ Hz, 2H), 8.18 – 8.08 (m, 4H), 8.03 (dd, $J = 8.4, 6.4$ Hz, 4H), 5.54 (d, $J = 3.6$ Hz, 1H), 4.76 – 4.51 (m, 2H), 4.08 – 3.98 (m, 4H), 3.49 (q, $J = 4.9$ Hz, 2H), 3.11 (s, 9H).

^{13}C NMR: (125 MHz, DMSO-d_6): δ 193.41, 165.26, 134.71, 134.46, 130.44, 130.33, 130.14, 72.79, 65.96, 58.80, 53.41, 53.67.

ESI-MS (positive mode): m/z calculated for $\text{C}_{24}\text{H}_{28}\text{NO}_{10}\text{P}$ 521.15; found $\text{C}_{24}\text{H}_{29}\text{NO}_{10}\text{P}^+$ 522.1.



¹H NMR of GPC-CHO (400 MHz, DMSO-d₆)



¹³C NMR of GPC-CHO (125 MHz, DMSO-d₆)

2.4.2.15 Synthesis of Boc-AP-LA

Boc-AP-LA was synthesized through reported procedure^[43].

2.4.2.16 Synthesis of AP-LA

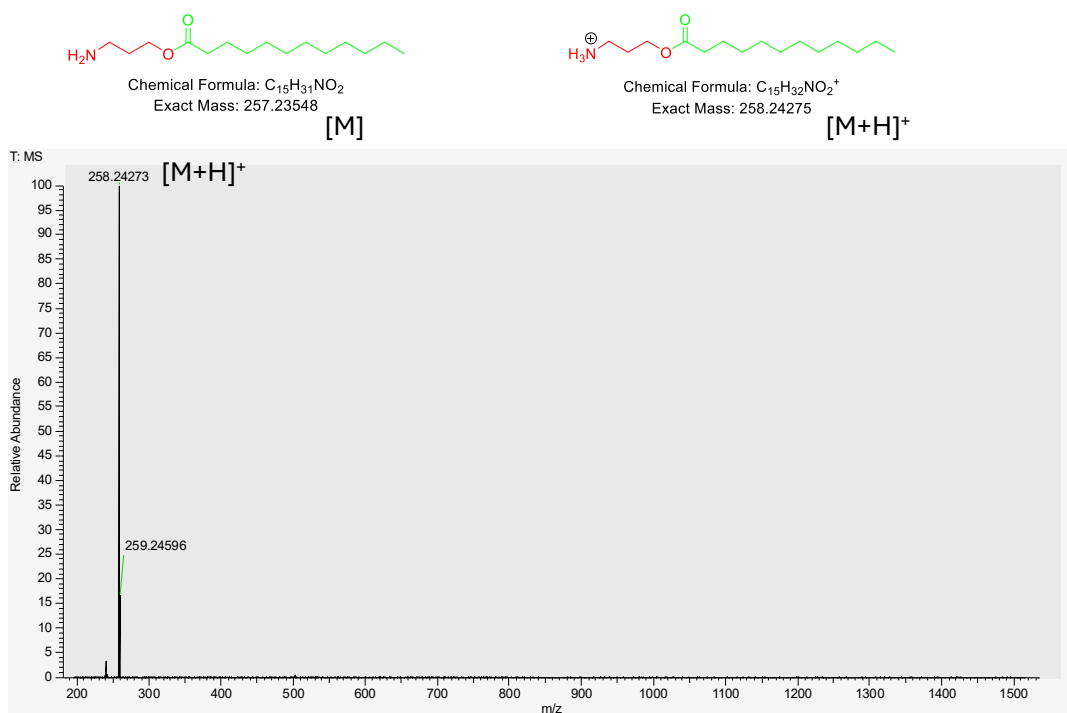
620 mg (1.73 mmol) of Boc-AP-LA was taken in a 100 mL round bottom flask dissolved in 5 mL of dichloromethane (DCM) and placed in an ice bath at 0 °C. Then 3 mL of TFA was added drop wise to the solution. After 15 minutes the ice bath was removed, and the reaction was stirred at room temperature for 4 hours. After 4 hours a thin layer chromatography (TLC) was performed to check the completion of the reaction. The solvent was then removed under reduced pressure and obtained as a light brown oil. 20 mL of cold diethyl ether was added to the brown liquid to obtain a white solid and the excess ether was decanted afterwards. The white solid was kept under high vacuum overnight to remove the remaining diethyl ether. The final product was obtained as off white flaky solid.

Yield: 600 mg (Quantitative Yield)

¹H NMR (400 MHz, DMSO): δ 7.90 (s, 3H), 4.07 (t, J = 6.3 Hz, 2H), 2.90 – 2.80 (m, 2H), 2.29 (t, J = 7.4 Hz, 2H), 1.92 – 1.81 (m, 2H), 1.56 – 1.46 (m, 2H), 1.24 (s, 17H), 0.89 – 0.82 (m, 3H).

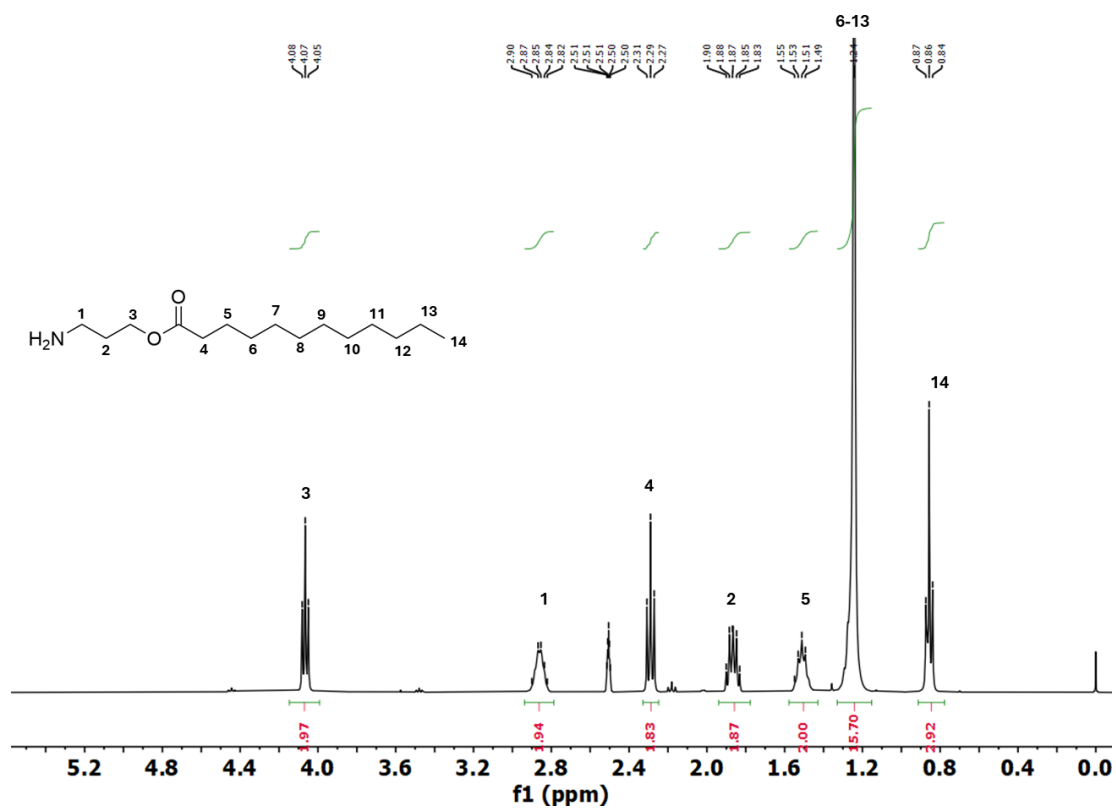
¹³C NMR (100 MHz, DMSO) δ 173.33, 61.41, 36.60, 33.84, 31.77, 29.47, 29.36, 29.18, 28.93, 26.84, 24.85, 22.56, 14.40.

ESI-MS (positive mode): m/z calculated for C₁₅H₃₁NO₂ 257.23; found C₁₅H₃₂NO₂⁺ 258.2.

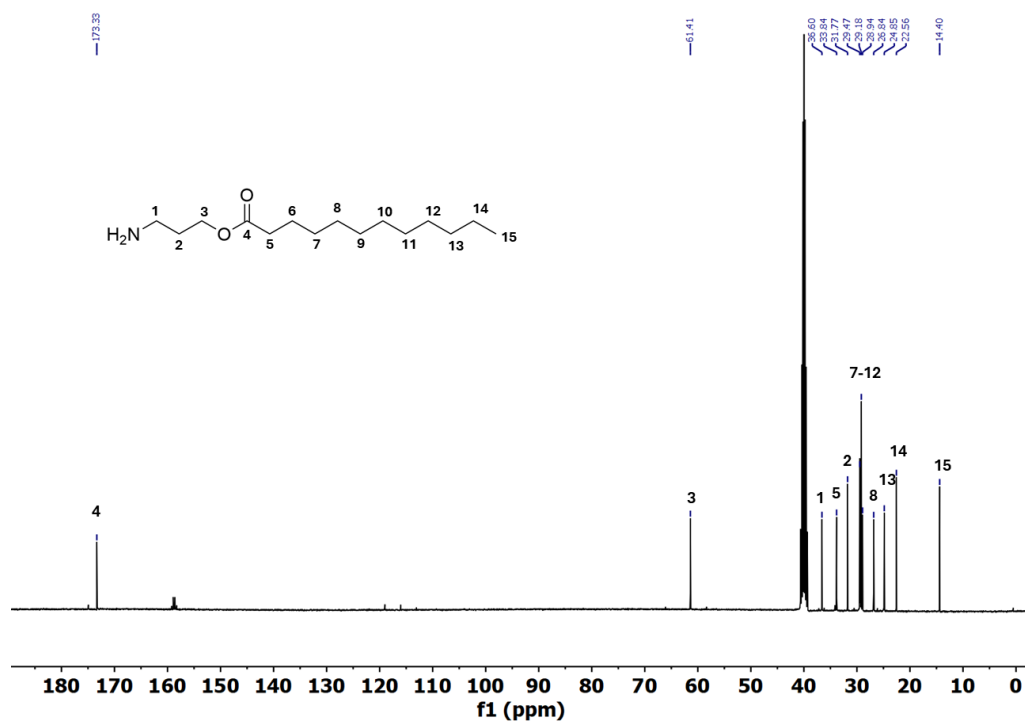


HRMS Spectra of AP-LA

HRMS (ESI, TOF): m/z found 258.24273; calculated for $C_{15}H_{32}NO_2^+ [M+H]^+ + 258.24275$, error = 0.08 ppm.



¹H NMR of AP-LA (400 MHz, DMSO-d₆)



¹³C NMR of AP-LA (100 MHz, DMSO-d₆)

2.4.2.17 Synthesis of BA-C9

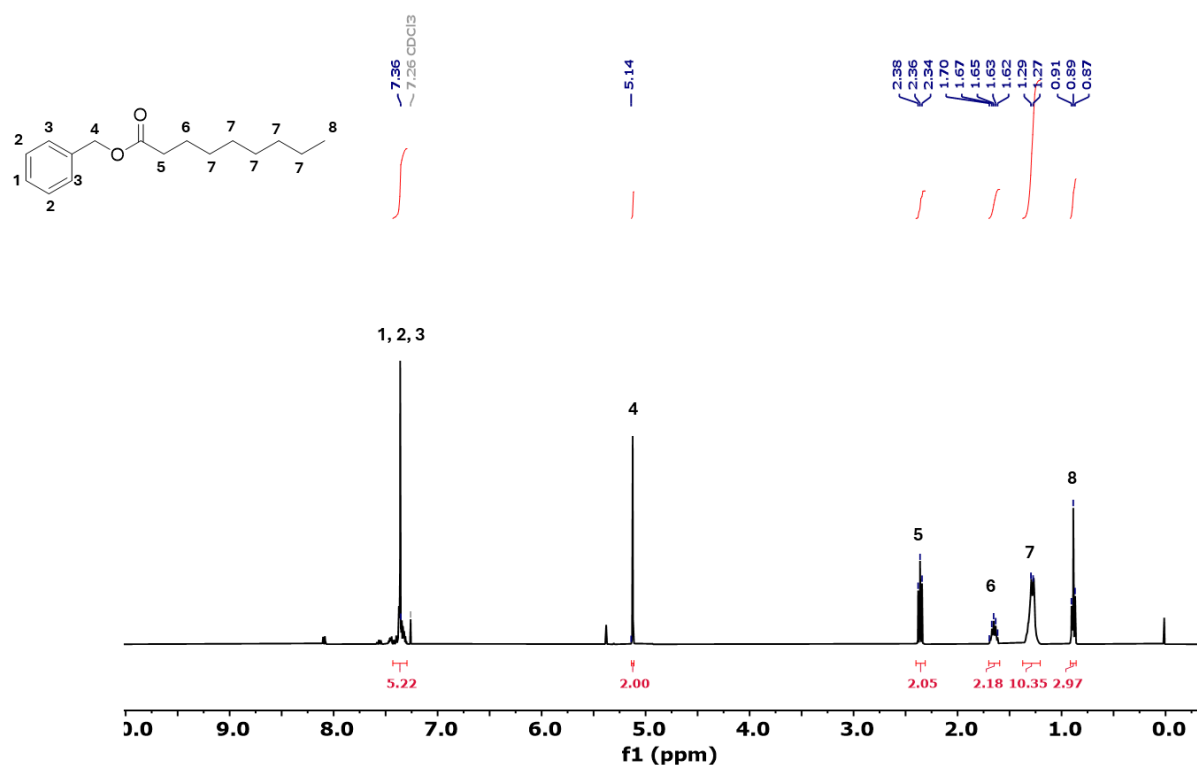
Benzyl alcohol (500mg, 4.62 mmol), nonanoic acid (1.09 g, 6.93 mmol), DCC (1.90 g, 9.25 mmol) and DMAP (565mg, 4.6 mmol) were dissolved in dry DCM solvent (25 mL) in a 100 mL round bottom flask and stirred overnight at room temperature. After the full consumption of alcohol, the reaction mixture was concentrated by evaporation on a rotary evaporator, and the concentrated crude mixture was purified by column chromatography (8:2 Hexane: EtOAc) on silica gel to obtain pure ester as light-yellow oil.

Yield: (925 mg) 81%

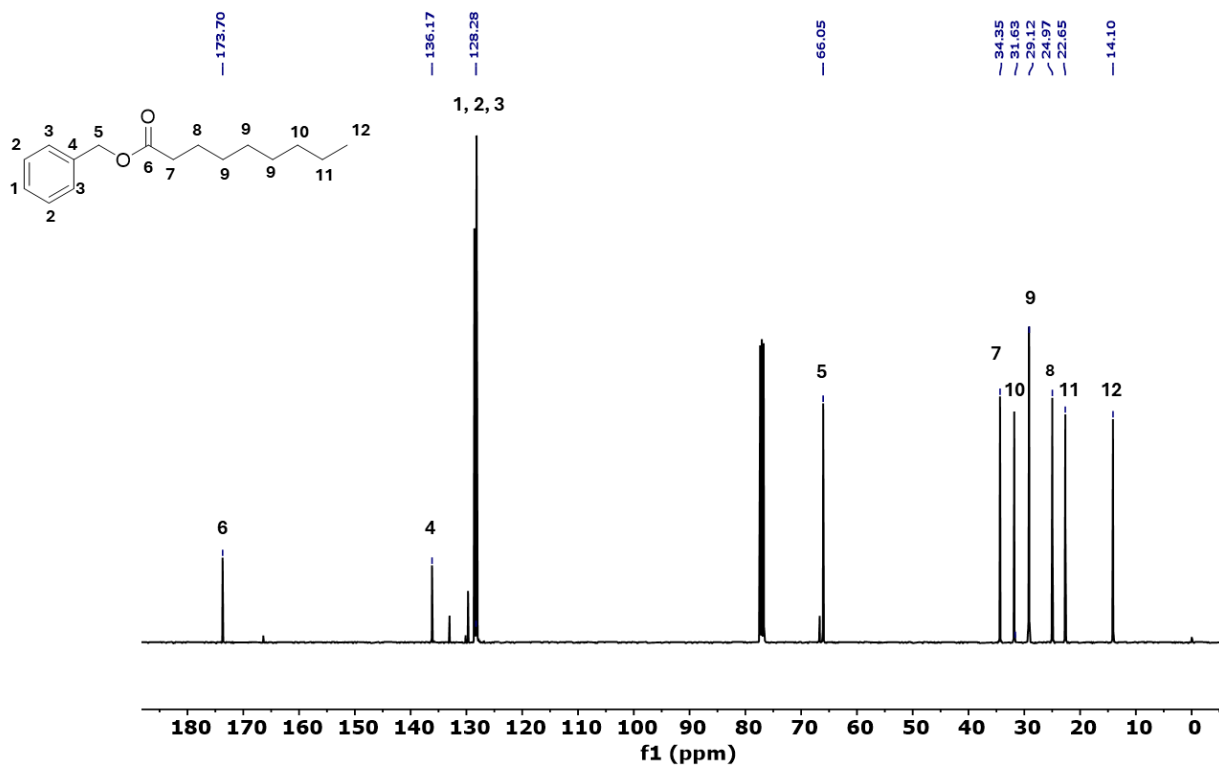
^1H NMR (400 MHz, CDCl_3): δ 7.36 (m, 5H), 5.14 (s, 2H), 2.34 (t, 2H), 1.62 (q, $J = 7.1$ Hz, 2H), 1.27 (d, 10H), 0.87 (t, 3H).

^{13}C NMR (100 MHz, CDCl_3): δ 173.70, 136.17, 128.28, 66.05, 34.35, 31.63, 29.12, 24.97, 22.65, 14.10.

ESI-MS (positive mode): m/z calculated for $\text{C}_{16}\text{H}_{24}\text{O}_2$ 248.18; found $\text{C}_{16}\text{H}_{25}\text{O}_2^+$ 249.2.



^1H NMR of BA-C9 (400 MHz, CDCl_3)



^{13}C NMR of **BA-C9** (100 MHz, CDCl_3)

2.4.2.18 Sample Preparation

Stock solutions of GPC-CHO (50 mM) and AP-LA (50 mM) were prepared in a 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer of pH 7.4. From these stocks, the respective working concentrations were made. The required amount of AP-LA fuel was injected into a vial containing GPC-CHO aldehyde in the buffer.

For sample containing lipase, required amount of GPC-CHO and lipase were mixed in the buffer solution and then AP-LA fuel was added and the whole solution was vortexed for 30 seconds. Stock solution of lipase (5.45mg/mL, 5777 Units/mL) was prepared in pH 7.4 HEPES buffer.

2.4.2.19 Nile red Localization and Release Studies

Stock solution of Nile red (10 mM) was prepared in acetone. From that 5 μL was transferred to a glass vial, and the solvent was evaporated under vacuum. The required amounts of stock solutions of GPC-CHO (1mM) and AP-LA (3mM) were added to the vial and mixed well to facilitate effective encapsulation of the Nile red. 200 μL of the Nile red encapsulated GPC-Imine was transferred to a 96 well-plate and an initial fluorescence intensity at 635 nm was measured using a Tecan Spark Plate reader (excitation wavelength = 545 nm). Different concentrations of lipase enzyme were

added and the changes in the fluorescence intensity over time was monitored at room temperature. We normalized the values with the initial intensity at 635 nm which served as the intensity before adding the enzyme.

2.4.2.20 Pyranine (HPTS) Encapsulation and Release Studies

To prepare pyranine (HPTS) loaded GPC-Imine liposomes, required amount of GPC-CHO (2mM) and AP-LA (6 mM) lipids were added to HEPES buffer containing 10 mM pyranine dye and allowed to self-assemble in situ. After equilibrating for 2 hours, the suspension was dialyzed for 24 hours using a D-Tube Dialyzer Maxi (MWCO 6 kDa) dialysis tube in fresh HEPES buffer to remove any unencapsulated dye. For the release study, the liposomes were diluted to a final concentration of 1 mM and transferred to a 96-well plate. Different concentrations of lipase enzyme were added to each well, and the release of HPTS was monitored by measuring the change in fluorescence at 515 nm (with excitation at 454 nm) by using Tecan Spark Plate reader.

2.5 Supporting Figures

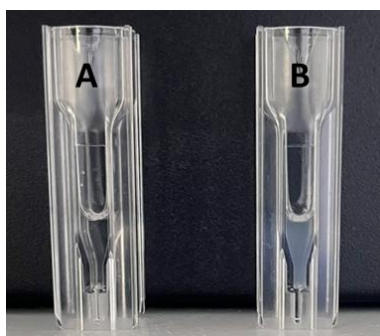


Figure S2.1. Self-assembly induced sample turbidity. The transparent solution of AP-LA (A) transforms into a cloudy solution (B) upon the addition of GPC-CHO at room temperature. [AP-LA] = 3 mM, [GPC -CHO] = 1 mM, pH 7.4 HEPES Buffer.

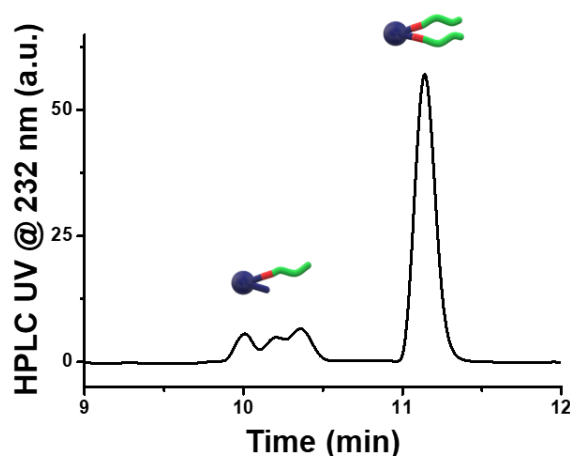


Figure S2.2: HPLC chromatogram 5 minutes after the addition of AP-LA to GPC-CHO. Multiple peaks at ≈ 10.19 minutes corresponds to single tailed GPC-Imine as detailed in Figure S4 and the peak having retention time 11.1 minutes corresponds to the double tailed GPC-Imine. (GPC-Imine was reduced with NaBH_3CN before injection into HPLC as described in HPLC experimental section).

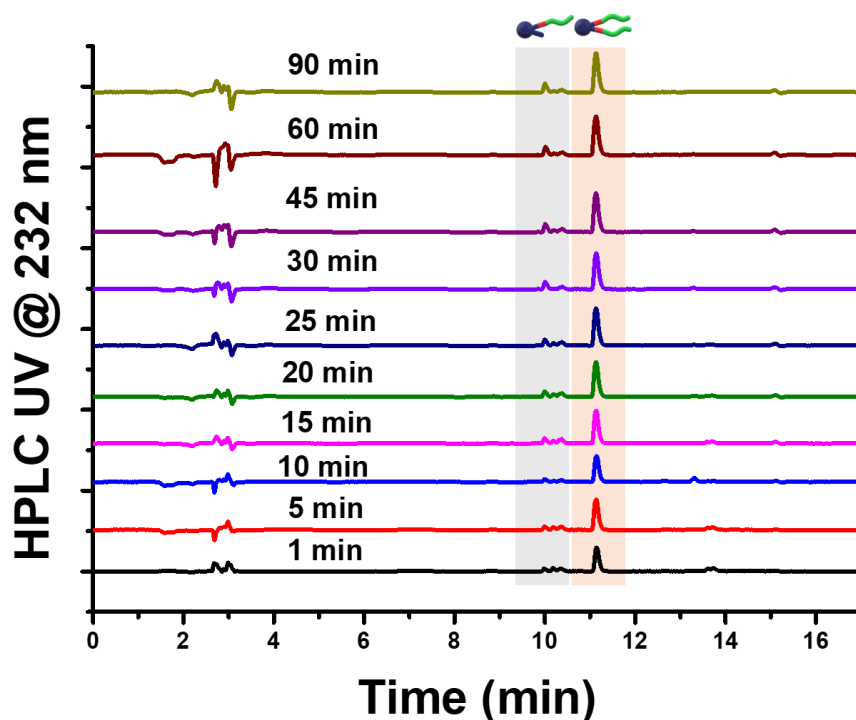


Figure S2.3: Time dependent HPLC chromatogram showing the formation of GPC-Imine along with single tailed GPC-Imine after reduction using NaBH_3CN . The peaks within 4 min are from the solvent front.

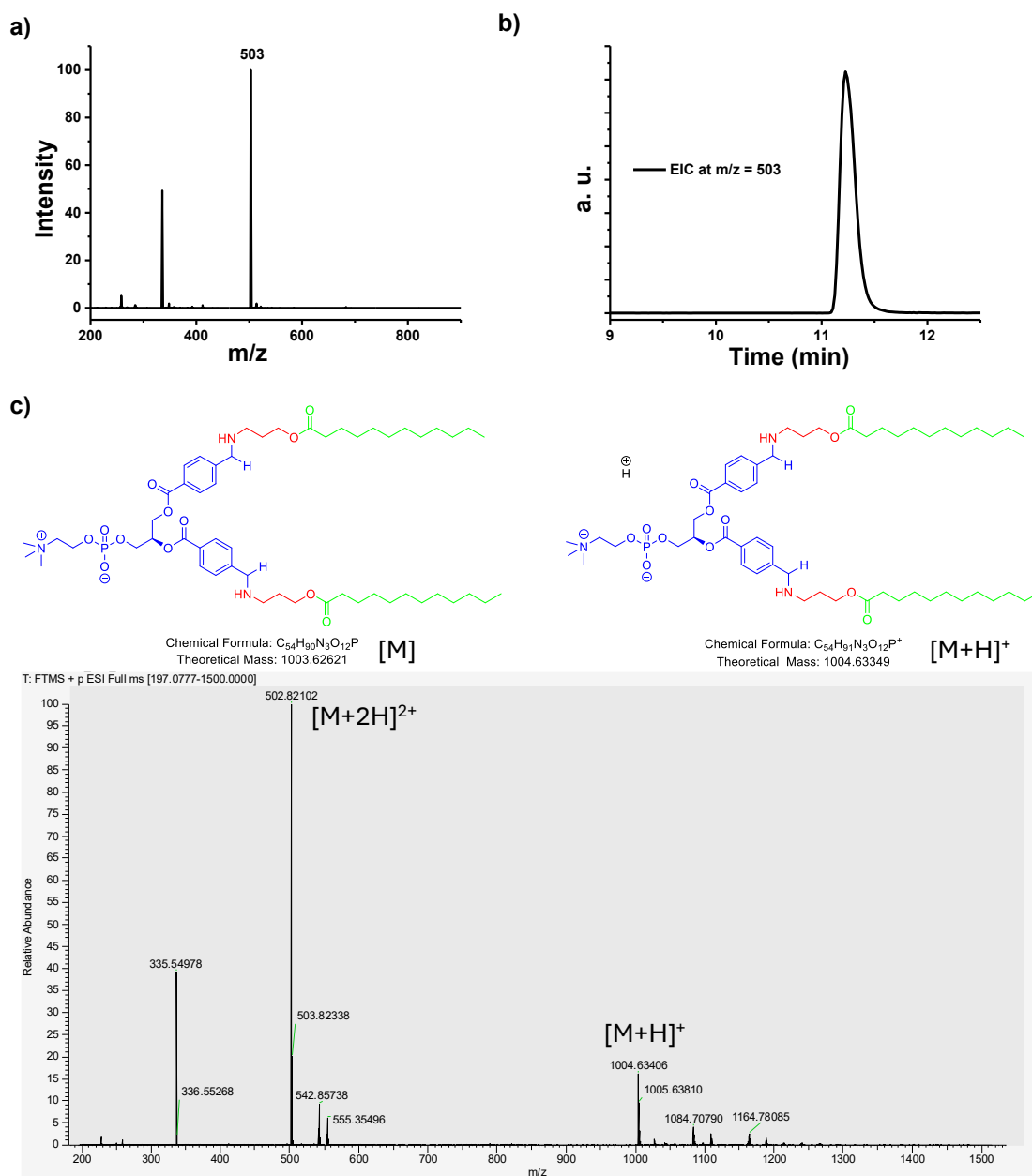


Figure S2.4: Mass spectrometry (MS) analysis a) MS data showing the $[M+2H]^{2+}$ ion of the GPC-Imine peak at retention time 11.1 minutes. b) Extracted Ion chromatogram (EIC) at $m/z = 503$. c) HRMS data of the reduced GPC-Imine. (GPC-Imine was reduced with $NaBH_3CN$ before injection into HPLC as described in HPLC experimental section).

HRMS (ESI, TOF): m/z found 1004.63406; calculated for $C_{54}H_{91}N_3O_{12}P^+$ $[M+H]^+$ + 1004.63349, error = 0.57 ppm.

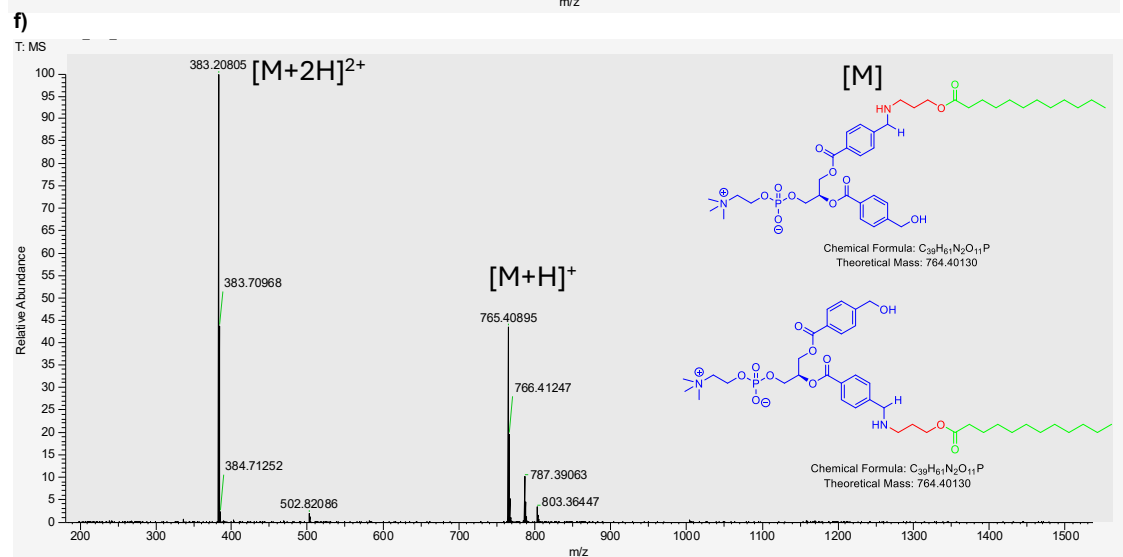
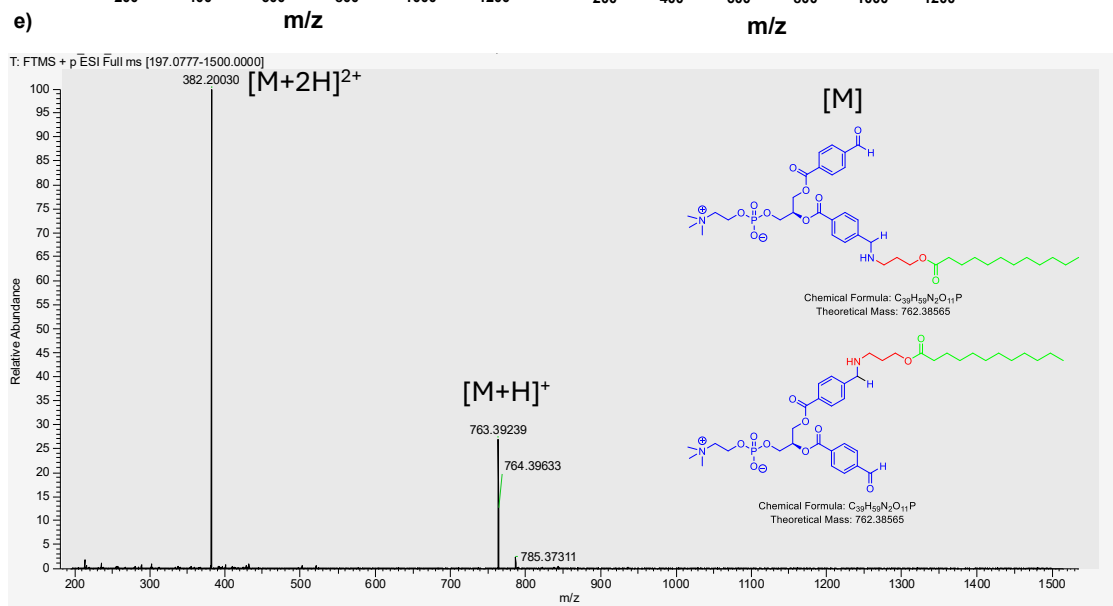
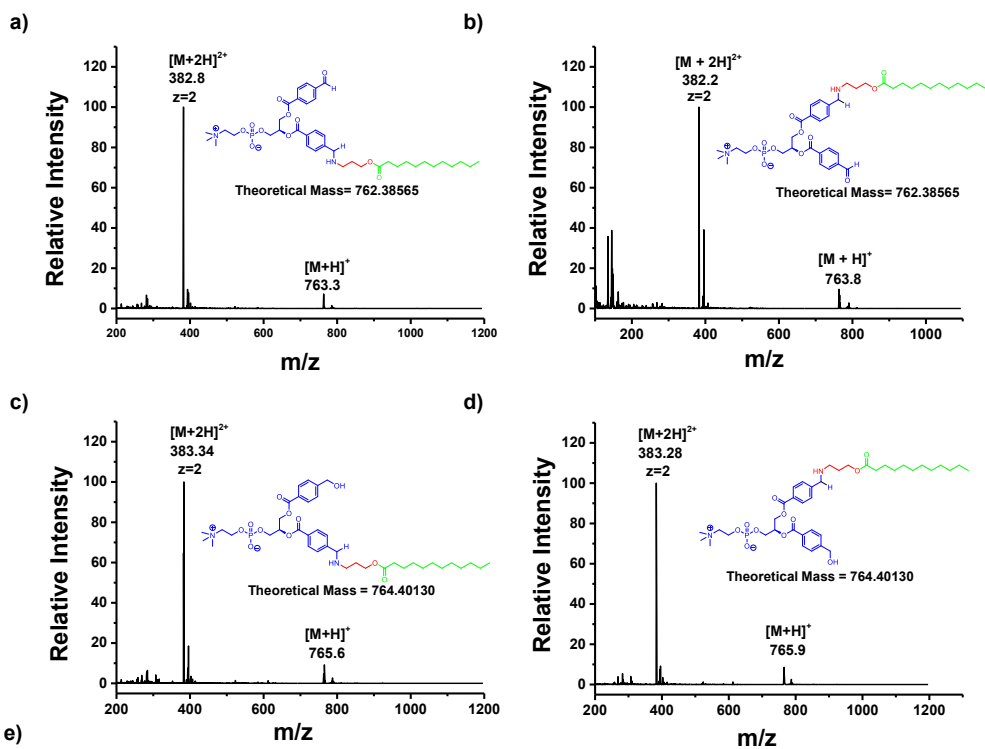


Figure S2.5: a-d) MS analysis and chemical structures of different single tailed possibilities due to the acylation of syn-1 and syn-2 positions leading to different stereoisomers and the reduction of free aldehyde upon addition of NaBH₃CN for HPLC analysis. e-f) HRMS data of the single tailed species.

HRMS (ESI, TOF): m/z found 763.39239; calculated for C₃₉H₆₀N₂O₁₁P⁺ [M+H] + 763.39347, Δ = -0.7 ppm.

HRMS (ESI, TOF): m/z found 765.40895; calculated for C₃₉H₆₂N₂O₁₁P⁺ [M+H]⁺ 765.40587, Δ = 0.5 ppm.

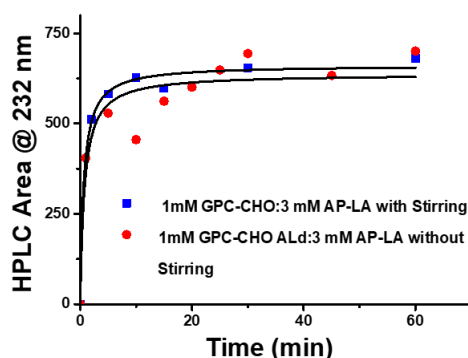


Figure S2.6: GPC-Imine formation kinetics with and without stirring through HPLC-MS showing similar formation kinetics. (GPC-Imine was reduced with NaBH₃CN before injection into HPLC as described in HPLC experimental section).

Note: Stirring the reaction mixture had negligible effects on the in situ GPC-Imine formation kinetics and therefore all other experiments were performed without stirring.

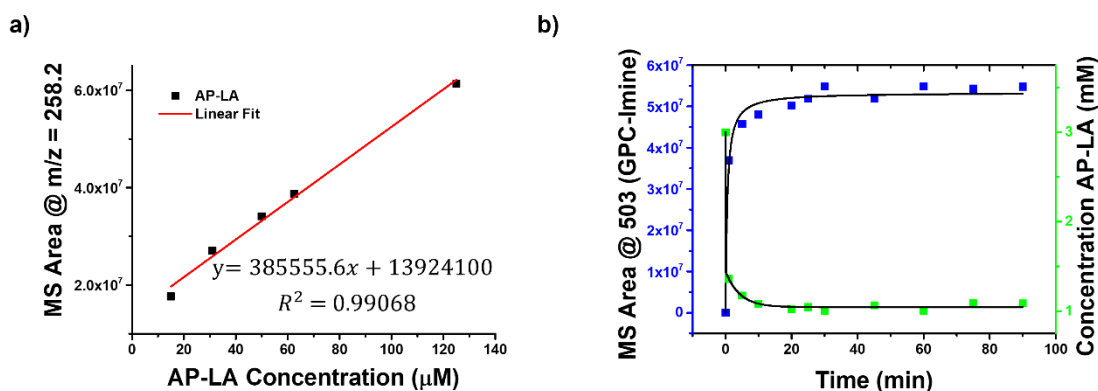


Figure S2.7: a) Standard calibration plot of AP-LA from MS. b) HPLC-MS kinetics showing the increase in MS area of GPC-Imine after reduction and decrease in the concentration of AP-LA.

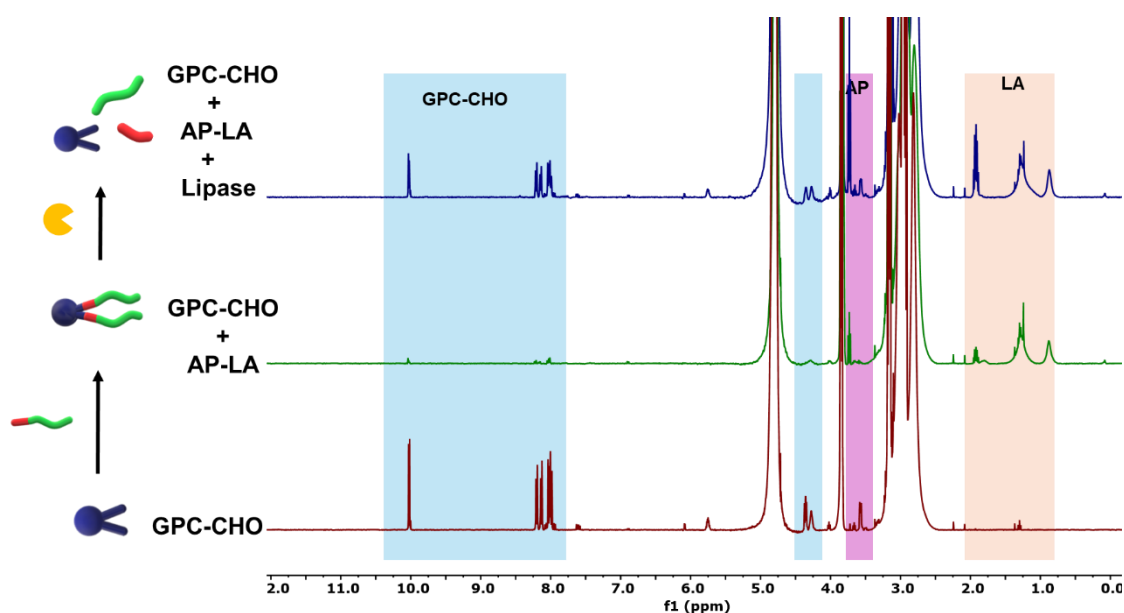


Figure S2.8: ^1H NMR showing the transformation of GPC-CHO after addition of AP-LA and Lipase. The aldehyde peak disappeared after the addition of AP-LA confirming the formation of GPC-Imine and its eventual self-assembly. Addition of lipase resulted in GPC-imine hydrolysis to regenerate GPC-CHO along with wastes as aminopropanol (AP) and lauric acid (LA). ^1H NMR of GPC-Imine before lipase addition shows no peaks confirming the complete consumption of GPC-CHO to form self-assembled GPC-Imine. After addition of lipase, we notice the emergence of peaks corresponding to GPC-CHO, AP, and LA which confirm the hydrolysis of GPC-Imine. Conditions: 5 mM GPC-CHO, 15 mM AP-LA and excess Lipase in 100 mM HEPES in D_2O , pH 7.5.

Note: We do not observe the formation of GPC-AP intermediate both in HPLC-MS and in NMR. This is because since GPC-AP is not amphiphilic enough to self-assemble, it cannot drive the equilibrium towards imine formation. Thus, the equilibrium shifts towards the hydrolysis of imine in GPC-AP to regenerate GPC-CHO along with wastes as aminopropanol (AP) and lauric acid (LA). Also, the new peak at aldehydic region is due to the presence of mixture of aldehyde and single side hydrated GPC-CHO.

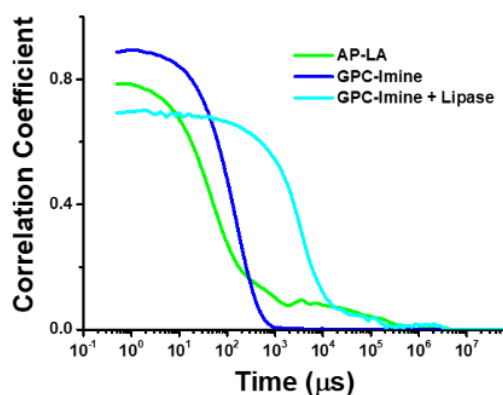


Figure S2.9: Correlation coefficient of different samples obtained from the DLS measurements. Smooth correlation coefficient of GPC-Imine confirms the presence of uniform sized self-assembled nanostructures.

Note: Although the correlogram after the lipase addition reveals that it is multimodal and thus not ideal for DLS size analysis, we have shown the size in the manuscript for getting an idea of the supramolecular transformation into large crystalline structure of waste LA.

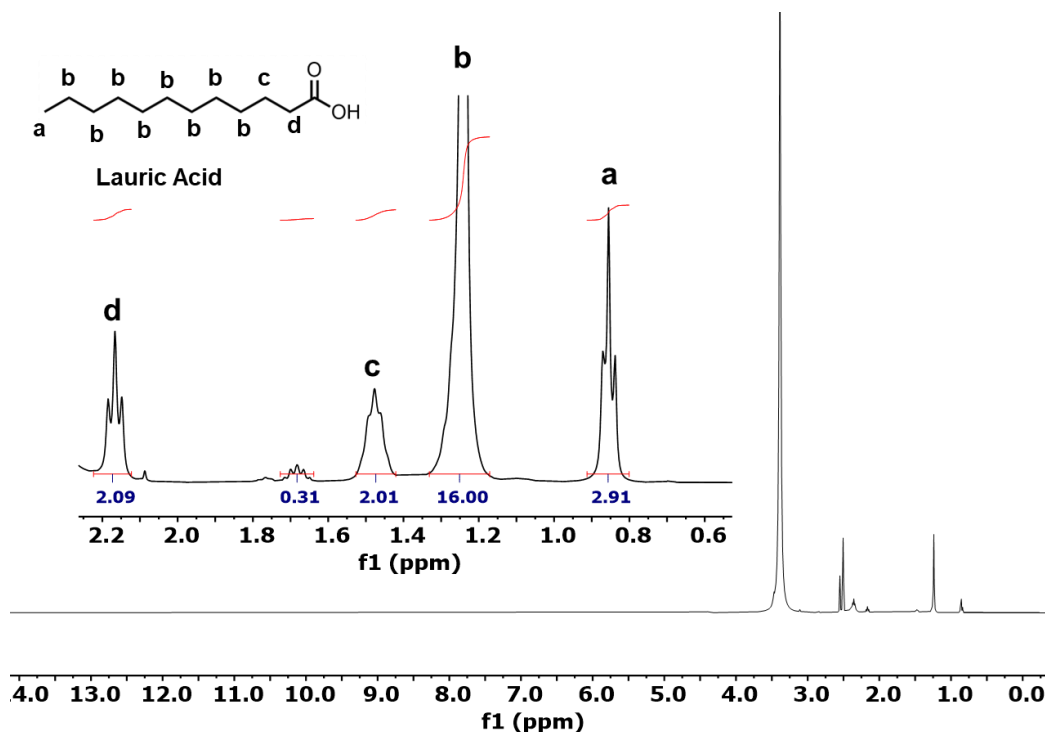


Figure S2.10: ^1H NMR (dmsO-d_6) of the crystals formed by the lipase activity. After the lipase activity, the precipitated crystals were separated by centrifugation. After drying the crystals ^1H NMR was performed in dmsO-d_6 . ^1H NMR signals confirmed the crystals precipitated are the crystals of the waste lauric acid (LA).

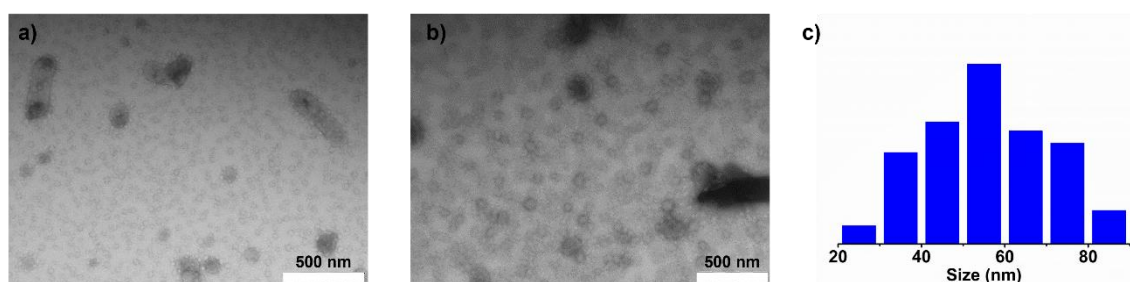


Figure S2.11: a) and b) additional TEM micrographs showing the GPC-Imine vesicles Scale bars: 500 nm. c) diameters of 230 vesicles measured manually using ImageJ software.

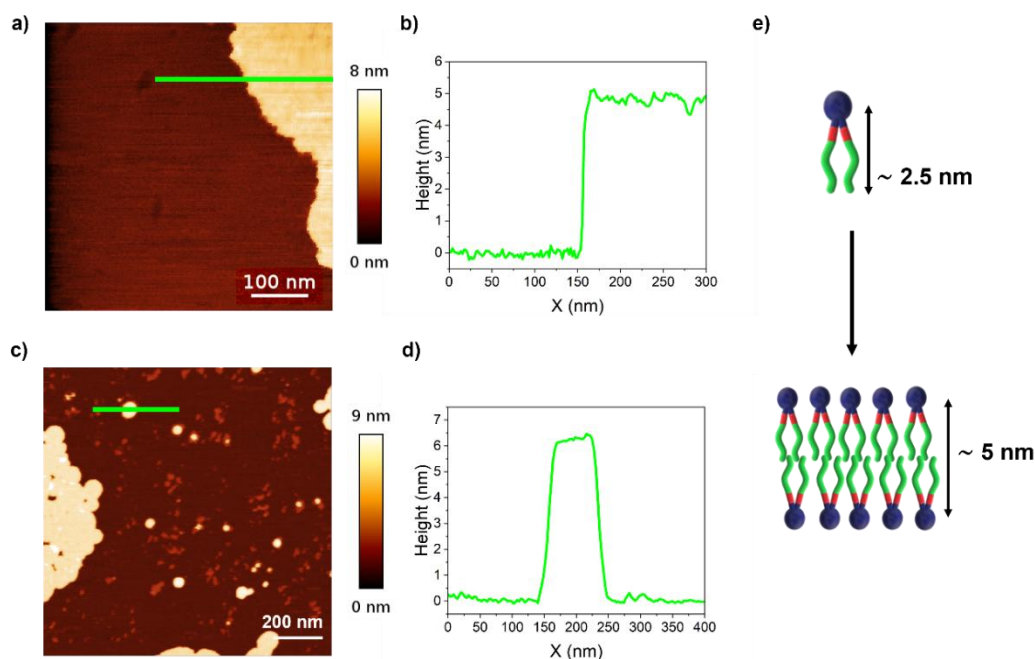


Figure S2.12: AFM topography of GPC-Imine. a) Image showing the formation of GPC-Imine lipid bilayer membrane on mica. b) AFM height profile of the bilayer membrane shows a thickness of 5 nm. c) AFM image of collapsed GPC-Imine vesicles with a size of roughly 100 nm and few smaller vesicles. This is higher than the TEM and DLS measurements probably because of the collapsing of vesicles on the surface. d) height profile across the collapsed vesicles of c) gives height = 6.5 nm. e) Schematic view of dimension and molecular organization of GPC-Imine and the GPC-Imine bilayer.

Note: In d) although collapsed vesicle should have shown a height which is double of b) i.e., approx. 10 nm. But we only observe a 6 nm height which could be due to further interdigitation of alkyl tail when they collapse on the surface. Also, it is possible that the scanning tip deforms the sample while imaging. Sample deformation effects may be more prominent in the case of collapsed or partially collapsed vesicles.^[44]

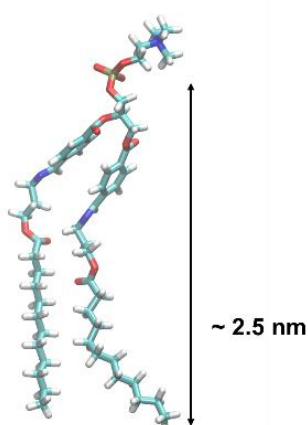
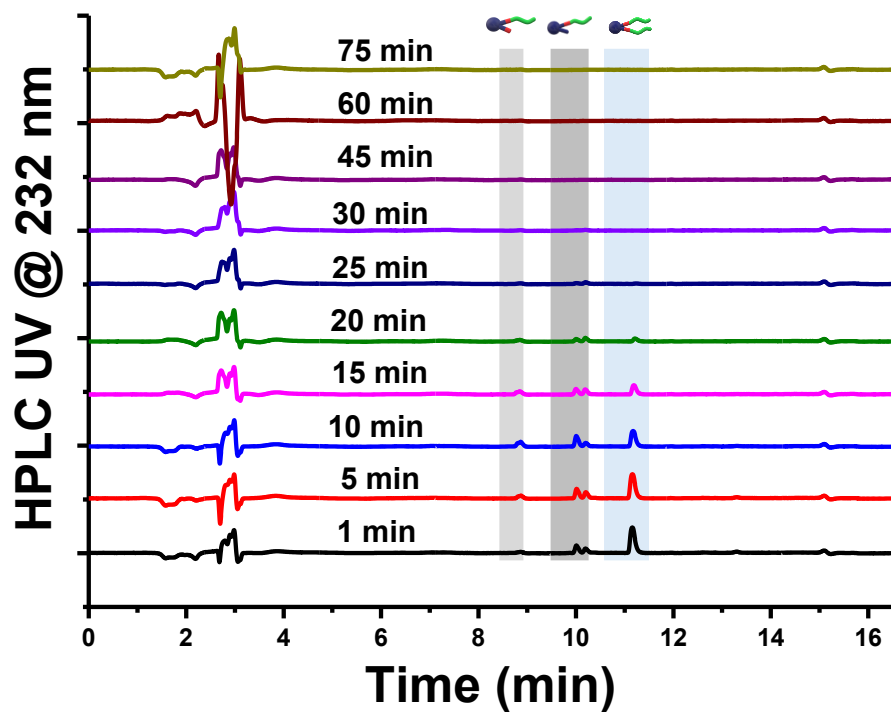


Figure S2.13: Energy minimized structure of GPC-Imine obtained from the DFT calculations.

Note: The GPC-Imine molecule was initially designed in ChemDraw and subsequently geometrically optimized at a DFT level with the B3LYP functional and the def2-TZVP basis set.^[45-48]

a)



b)

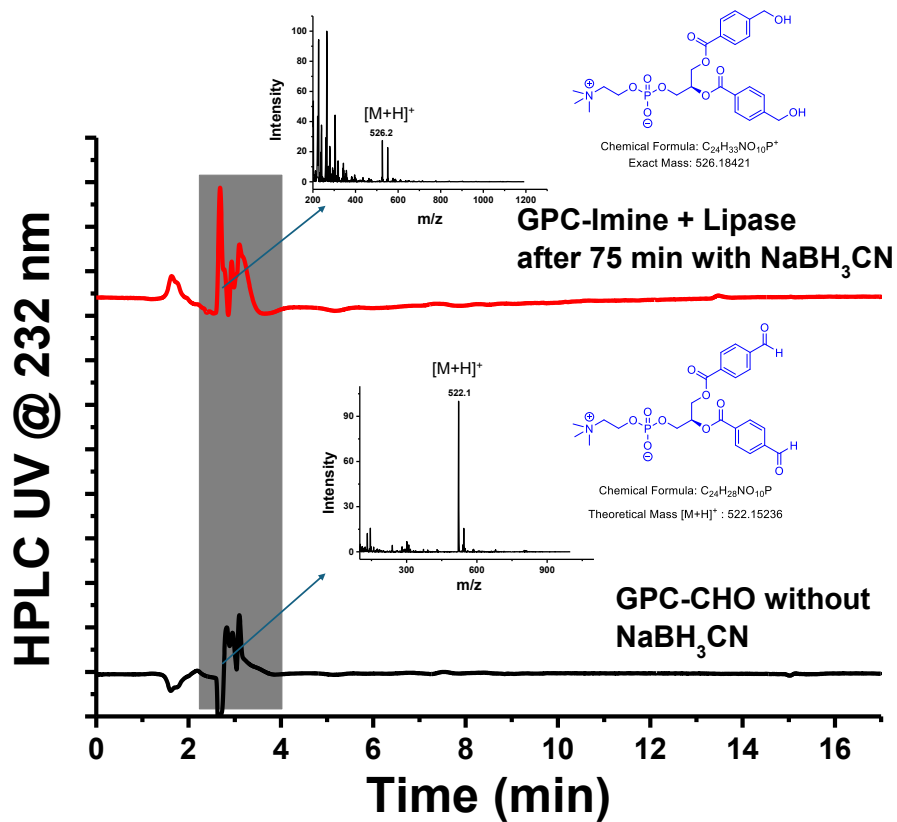


Figure S2.14: a) Time-dependent HPLC chromatograms in presence of 600 U/mL lipase showing the autonomous formation and degradation of GPC-Imine. b) The peaks within 4 min are from the solvent front. (GPC-Imine was reduced with NaBH_3CN before injection into HPLC as described in HPLC experimental section).

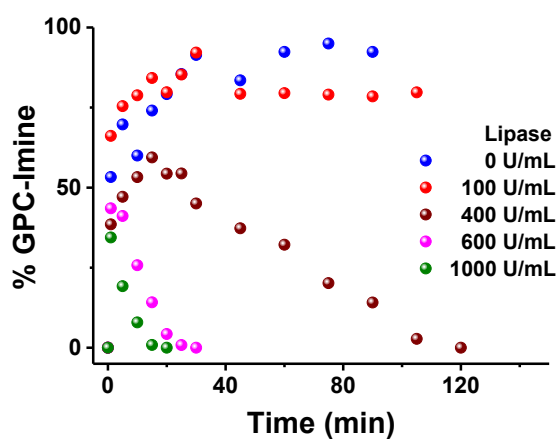


Figure S2.15: Time-dependent HPLC analysis in presence of lipase showing the autonomous formation and degradation of GPC-Imine resulting in transient lipid vesicles. (GPC-Imine was reduced with NaBH_3CN before injection into HPLC as described in HPLC experimental section).

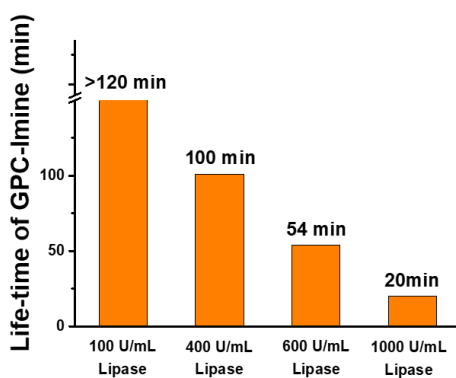


Figure S2.16: Bar diagram showing the lifetime of vesicles by changing the enzyme concentration.

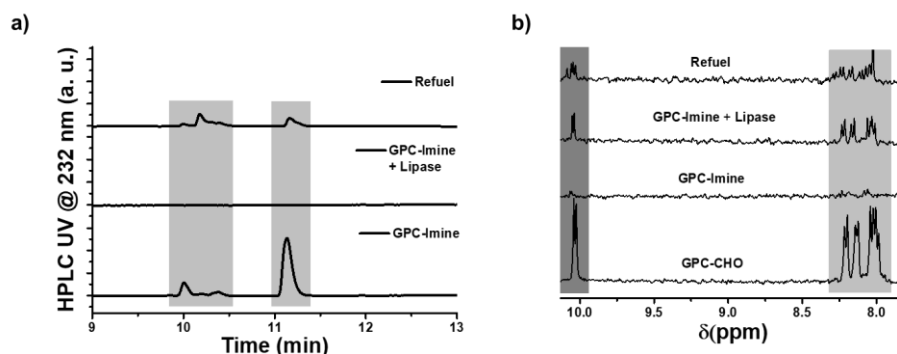


Figure S2.17: a) HPLC chromatogram showing the reformation of GPC-Imine after the addition of new batch of AP-LA (3mM) after the first cycle. b) ¹H NMR (100 mM HEPES in D₂O) of the refuelled system showing the decrease in the aldehyde and aromatic protons of the GPC-CHO after addition of new batch of 3mM AP-LA to the system after first cycle. (GPC-Imine was reduced with NaBH₃CN before injection into HPLC as described in HPLC experimental section).

Note: The NMR is taken roughly after 5 minutes after the addition of the subsequent batch of the fuel. Hence there can be the free GPC-CHO, single tailed imine and hydrated GPC-CHO (i.e. geminal diol upon reaction of aldehyde with H₂O) present in the solution as confirmed by LC-MS. The new peak in the aromatic region is due to this reason.

In Fig. S17a, there is no number and units on the vertical axis because these numbers have no absolute value and as we have stacked multiple chromatograms, it further loses its meaning. Thus, we have not included these values in the Y-axis.

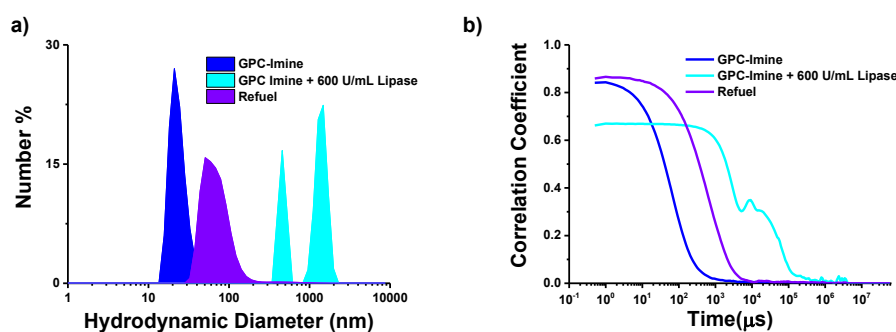


Figure S2.18: DLS number percent and correlation function of the refueled system representing the formation of GPC-Imine after the addition of new batch of fuel AP-LA. We notice that the vesicles after refueling are higher in size compared to the first cycle. Conditions: Addition of 3 mM AP-LA after first cycle containing 1mM GPC-CHO + 3mM AP-LA + 600 U/mL lipase.

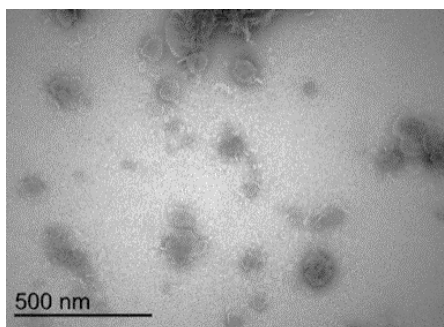


Figure S2.19: TEM image of the refueled system showing the multilamellar vesicles. Conditions: 1mM GPC CHO + 3mM AP-LA + 600 U/mL lipase + 3 mM AP-LA.

Note: Drop casted 10 μ L of refueled reaction mixture solution into the carbon grid after 5 minutes of the fuel addition.

Note: We hypothesized that the multilamellar vesicles are formed due to the increase in the hydrophobicity due to excess of waste Lauric acid (LA) generated in the system. This excess waste can co-assemble with the GPC-Imine and create multilamellar vesicles. To prove our hypothesis, we have added 3 mM of LA externally to the reaction mixture containing GPC-CHO and AP-LA and allowed it to form GPC-Imine vesicles. The DLS and the CLSM data (Figure S17-18) proved that the excess waste LA present in the system is co-assembling with the GPC-Imine and making the vesicles multilamellar.

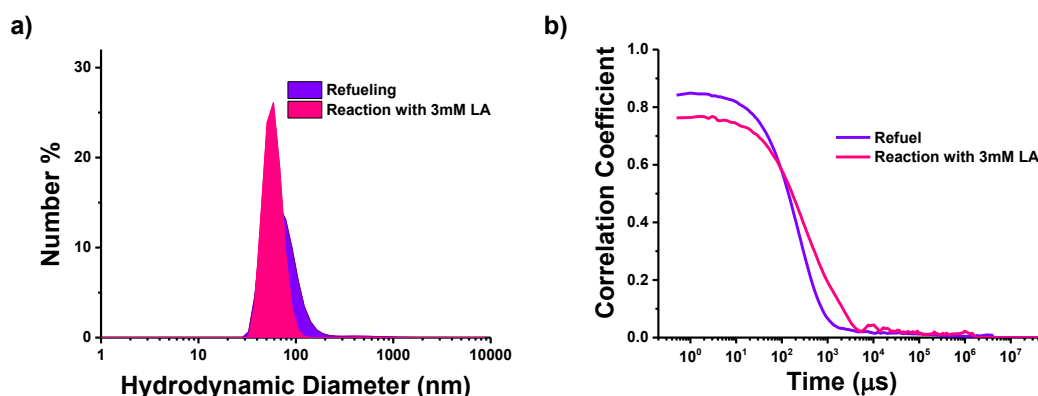


Figure S2.20: DLS a) Number percentage and b) Correlation co-efficient data showing the influence of lauric acid in the size of the GPC-Imine while refuelling. Conditions: 1mM GPC-CHO + 3mM AP-LA + 3 mM LA.

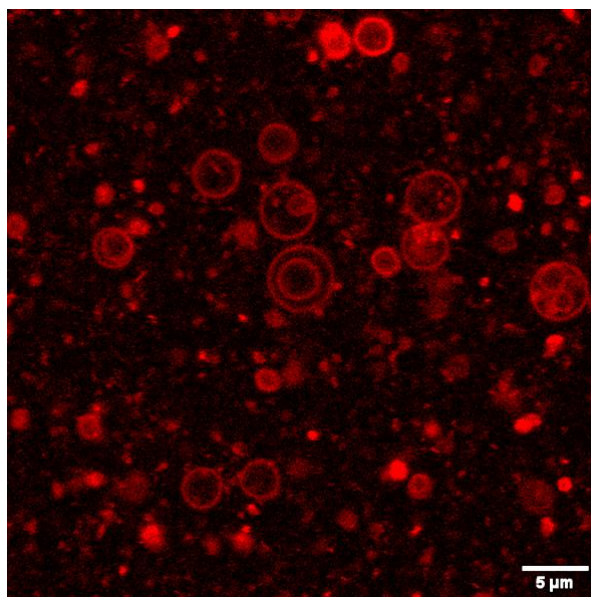


Figure S2.21: CLSM image of liposomes formed with 3mM lauric acid stained with 0.2 mol % Nile red. The formation of multilamellar vesicles with the addition of LA in the reaction confirmed the co-assembly of waste LA to GPC-Imine leading to the formation of multilamellar vesicles. Since this is a solution state imaging, it confirms the formation of vesicles in solution.^[49]

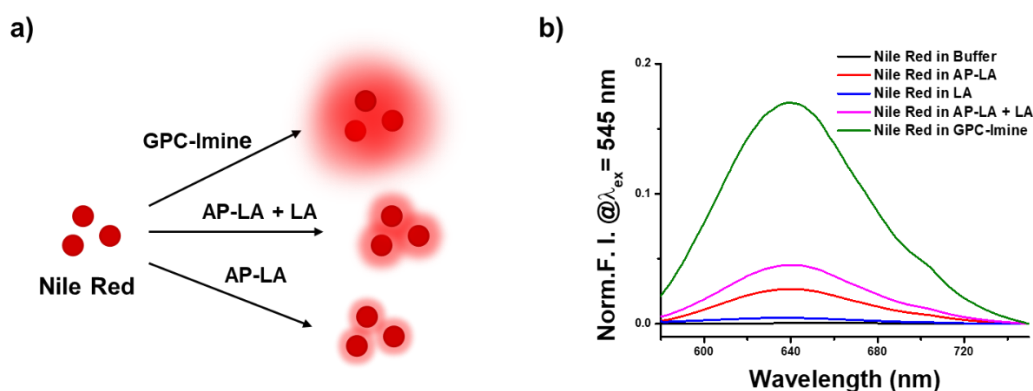


Figure S2.22: Schematics showing the change in fluorescence of Nile red when encapsulated in GPC-Imine, AP-LA and AP-LA + lauric acid. b) Normalized fluorescence spectra of Nile red indicate that higher intensity sample has hydrophobic pockets within supramolecular assembly like the bilayer interior of the vesicle. Conditions: 1mM GPC-Imine, 3mM AP-LA, 3 mM LA and 3mM AP-LA with 3 mM LA.

Note: We investigated if the physical interactions between the amino group of the fuel and the carboxylic acid group of lauric acid results in any self-assembly, using Nile Red as a fluorescent probe to monitor the self-assembly. We observed that the fluorescence intensity of Nile Red with AP-LA+Lauric acid sample (AP-LA + LA) is similar to the sample containing AP-LA alone. Furthermore, the emission intensity of AP-La + LA sample is ~70% lower than the ones obtained with GPC-Imine vesicle (Figure below). This indicates that even though there might be weak interaction between the lauric acid and AP-LA, it does not result in the formation of strong self-assembly.

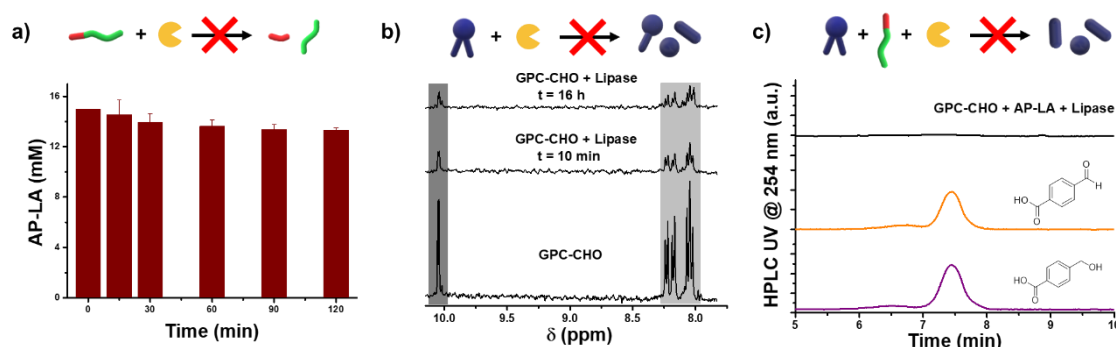


Figure S2.23: a) HPLC-MS analysis of the reactivity of lipase on the fuel AP-LA. Even after 2h of reaction there was no significant reduction of AP-LA concentration confirms the non-hydrolytic activity of lipase towards fuel AP-LA. b) ¹H NMR analysis of GPC-CHO with lipase over 16 h. The absence of new peaks suggested the lipase is unreactive to the ester bonds in the hydrophilic GPC-CHO which further confirms the selective interfacial lipase activity within vesicles only. c) HPLC chromatogram of the lipase treated solution of GPC-CHO, AP-LA and its comparison with different benzoic acid derivatives. The absence of any peak at retention time 7.5 minutes confirmed the inactivity of lipase on the ester bond present within the hydrophilic head GPC-CHO group. Conditions: 1000 U/mL Lipase.

Note: Since we are converting the unstable imine to amine for HPLC analysis using NaBH₃CN, if there was any ester hydrolysis of the GPC-CHO hydrophilic unit, the released carboxy benzaldehyde will be converted to its alcohol form in the presence of NaBH₃CN. Due to this reason, we have also converted the carboxy benzaldehyde into its alcohol form and injected into HPLC-MS to check whether we are having the lipase catalysed ester hydrolysis at the syn-1 and syn-2 positions. Our analysis confirms that lipase cannot hydrolyse GPC-CHO as well as AP-LA and it selectively hydrolyses self-assembling GPC-Imine.

For the experiment of Fig. S2.23a, we observed that the AP-LA molecules undergo a side reaction, i.e. intramolecular attack of terminal amine to the ester, to result in an amide with an alcohol in the terminal. This reaction happens even without the presence of Lipase, indicating it does not need a catalyst. However, the side reaction is limited to <7% of the total fuel in 2 hrs, which is further suppressed in the presence of GPC-CHO (as AP-LA is consumed for the formation of GPC-Imine). Thus, this minor side reaction does not influence our experiment significantly.

In Fig. S2.23c, there is no number and units on the vertical axis because these numbers have no absolute value and as we have stacked multiple chromatograms, it further loses its meaning. Thus, we have not included these values in the Y-axis.

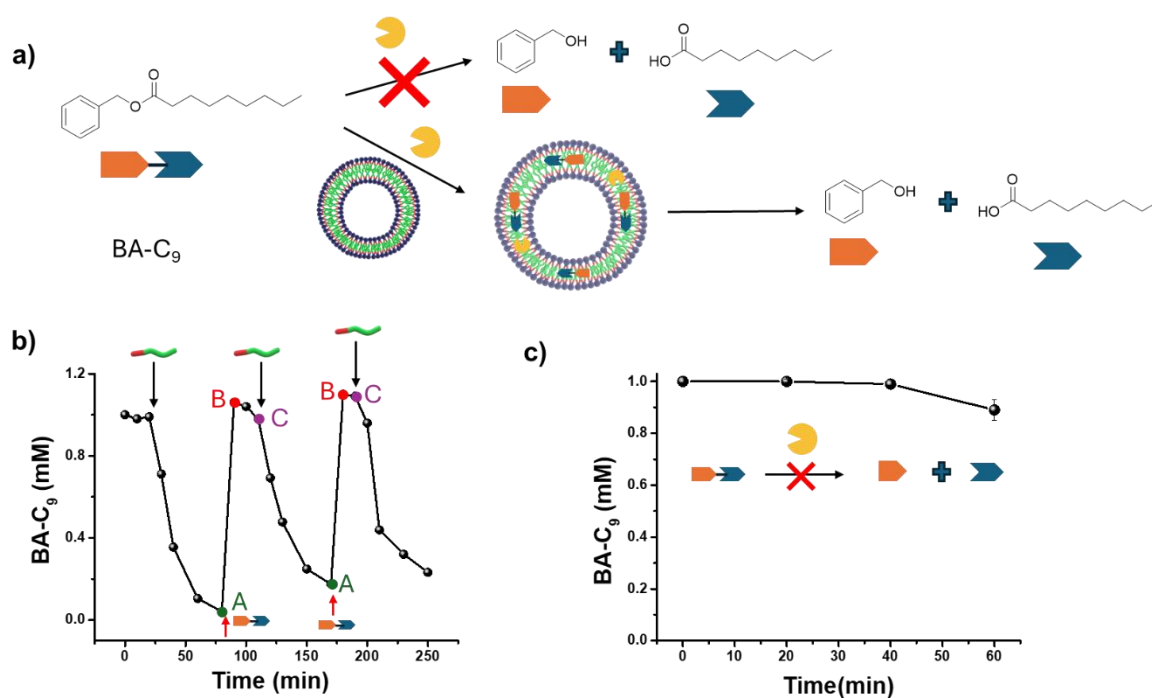


Figure S2.24: a) Schematic showing the sequestration of hydrophobic ester, Benzyl Nonanoate (BA-C₉), and its hydrolysis in presence and absence of GPC-Imine vesicles. b) HPLC analysis of hydrolysis of BA-C₉ over successive refuelling of the system, black arrow indicates the addition of fuel AP-LA and red arrow indicates the addition of ester BA-C₉. As can be seen that at point (A), there are no GPC-Imine vesicles present in the solution. When fresh BA-C₉ ester is added, it is not being hydrolysed (or slowly hydrolysed) between point (B) and (C). Hydrolysis is re-initiated upon re-formation of GPL-Imine by the addition of fresh fuel. c) HPLC analysis of the reactivity of lipase on the ester BA-C₉ over time in the absence of GPC-imine vesicle.

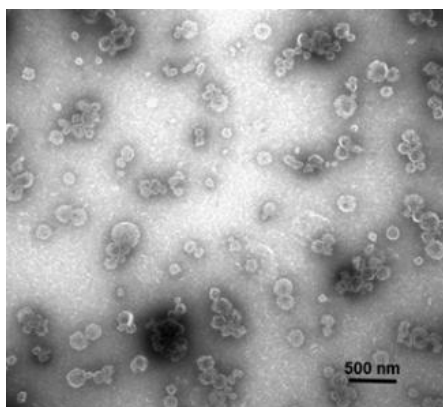


Figure S2.25: TEM micrograph of phospholipid vesicles at sustained assembly state (9 equivalent AP-LA and 600 U/mL Lipase enzyme).

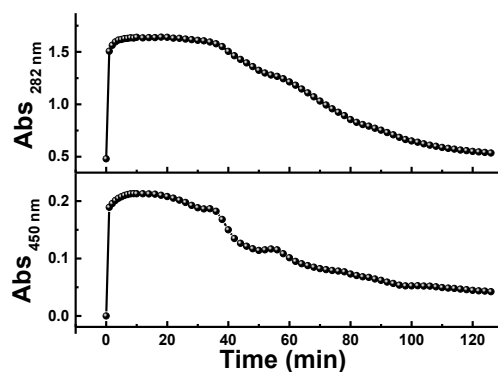


Figure S2.26: Simultaneous monitoring of absorbance at 282 nm and scattering at 450 nm shows that the unusual drop of absorbance at 40 min is due to the sedimentation of the lauric acid crystals. This clarifies the unusual drop in absorbance observed in Figure 3b, d, e of the main manuscript.

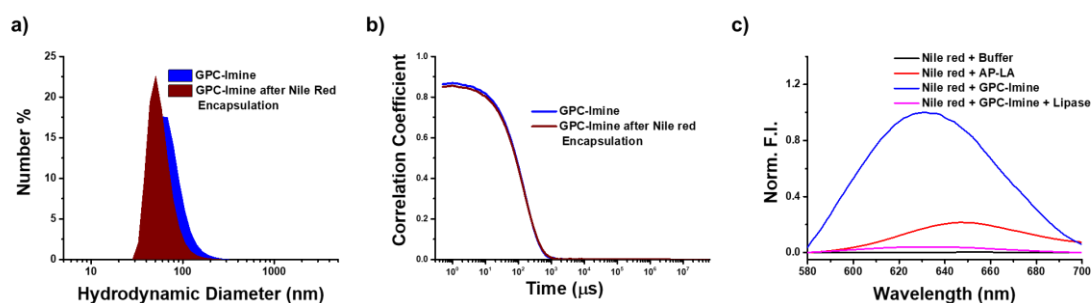


Figure S2.27: DLS a) Number percent and b) Correlation coefficient of GPC-Imine before and after localization of Nile red showing that the GPC-Imine vesicles are structurally intact after the incorporation of the dye. c) Normalized fluorescence intensity scan of Nile red showing the changes in the chemical polarity.

Note: The maximum emission wavelength of Nile Red blue-shifted from 648 nm in AP-LA to 637 nm in GPC-Imine confirming the incorporation of the dye in the GPC-Imine lipid hydrophobic pockets.

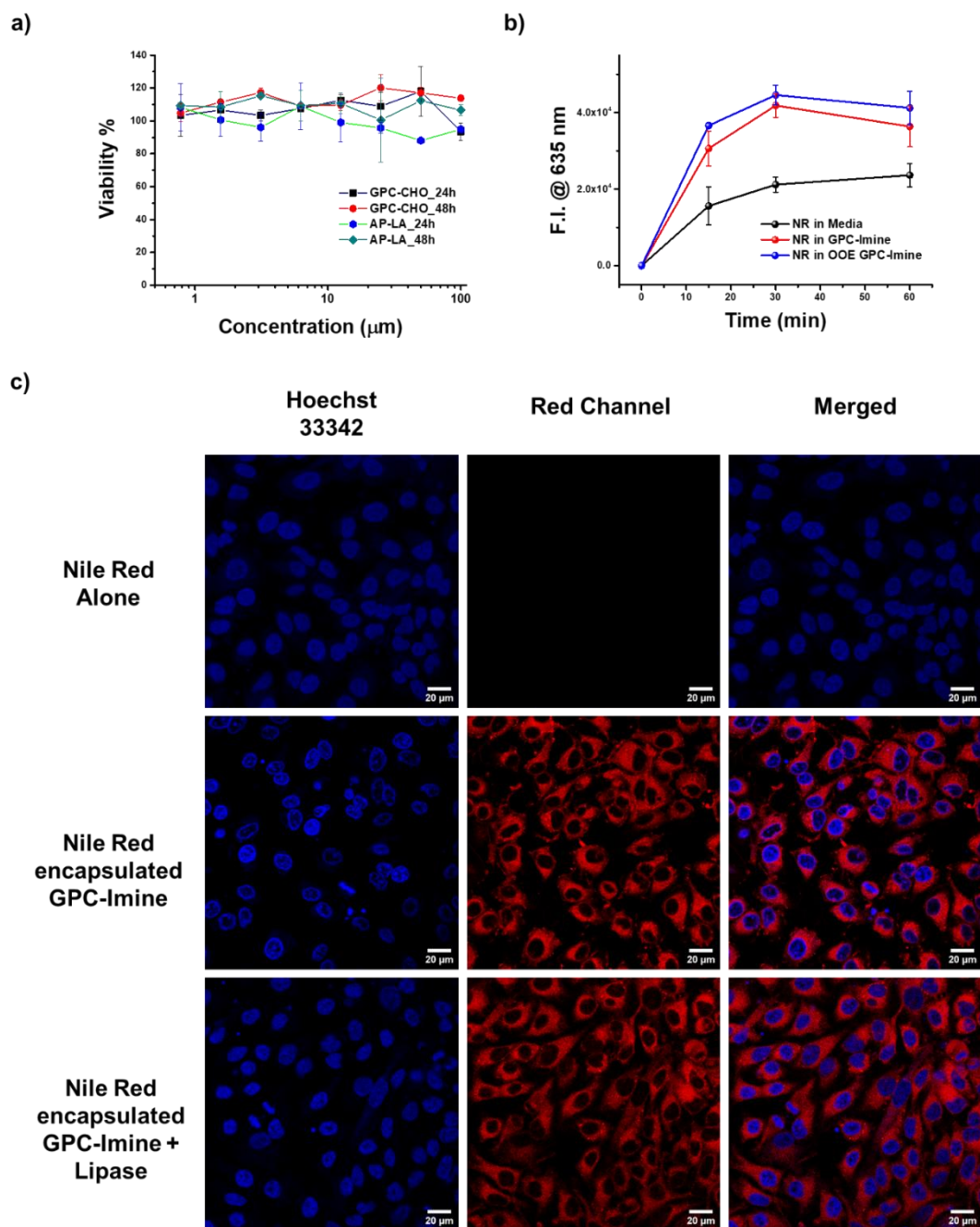


Figure S2.28: a) Cell Viability studies of the GPC-CHO and Fuel AP-LA. b) Nile red assay presenting the time dependent change in the emission of Nile red, showing the amount of Nile red delivered to the HeLa cells. c) Confocal images showing the release of Nile red to the HeLa cells after incubating each of the samples for 30 min.

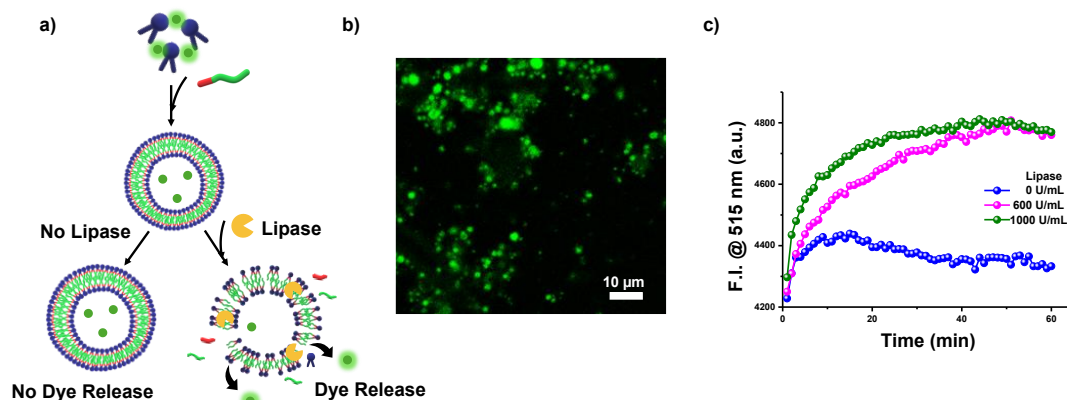


Figure S2.29: a) Schematic showing the encapsulation of hydrophilic pyranine dye and its release in presence and absence of lipase. b) Confocal images of pyranine encapsulated GPC-Imine vesicles. c) Release profile of pyranine dye from the GPC-Imine vesicles in presence of different concentration of lipase.

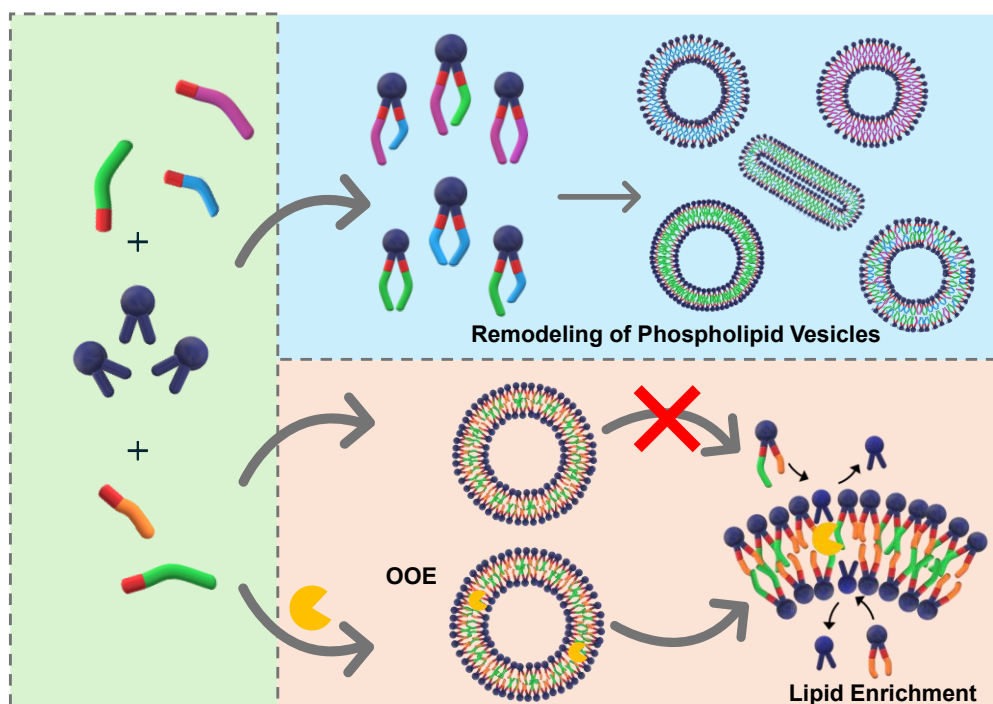
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Chapter 3. Fuel Selection and Remodeling of Synthetic Out-of-Equilibrium Phospholipid Vesicles



3.1 Introduction

One of the defining features of living cells is their remarkable plasticity, the ability of their membranes to continuously remodel in response to environmental and intracellular cues. This plasticity arises from a balance of lipid synthesis and degradation, processes that are inherently OOE and tightly coupled to cellular metabolism. From the structural perspective, plasticity arises because membranes are not static structures, their composition, curvature, and physical state are constantly tuned by enzymatic activity like of phospholipase, fuel consumption (ATP), and feedback from signalling pathways.^[1] Phospholipid remodeling pathways such as the Lands' cycle dynamically regulate bilayer composition to maintain membrane integrity, modulate fluidity, and enable processes like vesicle trafficking, endocytosis, and cytokinesis.^[2] It also allows cells to sense and respond to stimuli, maintain homeostasis, adapt to stress, and divide. Without continuous remodeling, membranes would lose their functional flexibility, and cells would be unable to sustain life.^[3-4]

Mimicking this level of complexity in synthetic systems has been a longstanding challenge. Classical lipid vesicles assembled at equilibrium capture compartmentalization but lack the dynamic turnover and remodeling intrinsic to biological membranes.^[5] Recent advances in OOE supramolecular chemistry have begun to address this limitation, demonstrating how chemical fuels can drive transient nanostructures similar to living materials.^[6-8] However, in living cells, phospholipid biosynthesis is a highly orchestrated process that depends on multiple enzymes and the continuous supply of different fatty acids. The enzymes couple the fatty acids to polar headgroups yielding the dialkyl lipids that constitute the bilayer membranes. Replicating this level of precision outside biology remains a formidable challenge, while efforts have focused on lipid formation and self-assembly^[9-14], the dynamic metabolic processes that govern lipid remodeling and turnover in native membranes are still poorly captured in synthetic systems.

In chapter 2 we have shown the example of chemically fueled, OOE synthesis of lipid vesicles that are inspired by natural membrane metabolism.^[15] However, we have shown the metabolic reactions of lipids vesicles with AP-LA fuel having fixed carbon chain length (12 carbons). But in living cells, there occurs a plethora of lipids where the cell uses lipids of varying chain lengths depending upon the need to perform specific function.

Despite these advancements, a true OOE system where tails are continuously remodeled as seen in natural cell membranes has not been achieved so far. This work demonstrates, for the first time, OOE mixed-tail lipid synthesis and their remodeling, providing a minimal model for kinetic remodeling processes akin to those operating in biological membranes.^[16] By linking hydrophobicity, kinetics, and composition, these results establish a framework for how synthetic systems can be programmed to undergo lipid remodeling under dissipative conditions, bringing us

closer to constructing artificial membranes that not only compartmentalize but also evolve dynamically, as living membranes do.

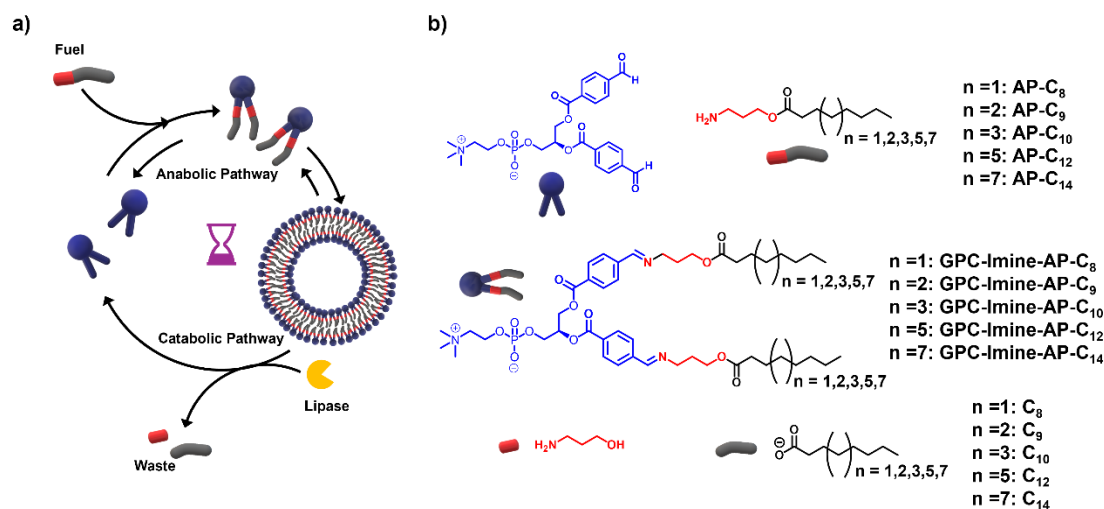


Figure 3.1. a) Schematics representation of in situ formation of phospholipids with temporal control. b) Chemical structures of the various fuels and components involved in the metabolic cycle.

3.2 Results and Discussion

The hydrophobic fuels with varying chain length were synthesized through the esterification of aminopropanol (AP) with aliphatic acids having carbon from C₈-C₁₄ (Figure 3.1, Scheme S3.1). As shown in chapter 2, the amine group of the hydrophobic fuels (AP-C₈, AP-C₉, AP-C₁₀ and AP-C₁₄) couples with the two aldehyde groups of the hydrophilic GPC-CHO, via imine bond, to form amphiphilic GPC-Imine.

The dynamic imine bond formation was investigated by adding 3 molar equivalents of each fuel to a solution of 1 equivalent of GPC-CHO at physiological pH (pH 7.4, 100 mM HEPES buffer). With AP-C₈ and AP-C₉, negligible changes in solution transparency were observed, indicating minimal self-assembled structure formation. In contrast, the reaction mixtures with AP-C₁₀, AP-C₁₂, and AP-C₁₄ became turbid within minutes, signifying the formation of self-assembled nanostructures. The in situ formation of self-assembling GPC-Imine lipids for each fuel was analysed using HPLC-MS after allowing the reaction mixtures to equilibrate for 2 hours, which confirmed imine formation in all cases (Figure 3.2a and Figure S3.1). Moreover, HPLC-MS analysis showed that fuels with longer alkyl tails, such as AP-C₁₀, AP-C₁₂, and AP-C₁₄, yielded higher percentage of imine formation compared to fuels with shorter alkyl tails like AP-C₈ and AP-C₉ (Figure 3.2b). This indicates the utility of different carbon chain length fuel in tuning the properties of vesicle formation.

Our earlier studies demonstrated that the red shift and disappearance of the 282 nm shoulder in the UV spectrum correspond to the conversion of GPC-CHO to GPC-Imine. Thus, we employed UV-Vis absorption spectroscopy to monitor imine formation using fuels with varying carbon chain

lengths. For GPC-Imine-AP-C₈, the spectra closely resembled those of GPC-CHO. Only a nominal shift in the absorption maxima and shoulder peak was observed, suggesting minimal formation of GPC-Imine with AP-C₈. In contrast, GPC-Imine-AP-C₉ exhibited a red shift compared to GPC-Imine-AP-C₈, along with partial disappearance of the shoulder peak, indicating increased imine formation with AP-C₉. For fuels with longer alkyl tails, the absorption maxima clearly red-shifted to 260 nm, and the shoulder at 282 nm flattened out completely, further validating the formation of GPC-Imine with longer alkyl tail fuels (Figure 3.2c).

We probed the kinetics of formation of GPC-Imine by monitoring the time dependent increase in the absorption at 282 nm upon addition of respective fuels (Figure 3.2d). The results revealed that the imine formation was directly linked to the chain length of the fuel used. We observed a slow formation of GPC-Imine with AP-C₈ and AP-C₉ fuels which did not saturate even after 1h, whereas rapid imine formation was observed with AP-C₁₂ and AP-C₁₄ fuels with plateauing of the signal within 20 minutes. The formation of GPC-Imine-C₁₀ displayed an intermediate kinetic response when compared to the longer chains by plateauing of signal over 60 minutes showing the formation over 60 minutes. These trends are consistent with the hydrophobic effect, a fundamental driving force in the self-assembly of amphiphilic molecules. Longer hydrophobic tails promote stronger interactions among hydrophobic regions and lead to fast and more stable aggregate formation. Furthermore, longer hydrophobic tails, minimizing unfavourable interactions with the aqueous environment leading to decrease in the system's free energy.^[17] This lowering of the system's free energy stabilizes the assemblies suggesting higher rate of forward reaction. Overall, the results confirms that GPC-Imine formation is closely linked to the self-assembly propensity of the fuels and is directly proportional to the carbon chain length of the fuels.

To further validate this trend, we carried out CAC measurements using Nile red as a polarity-sensitive probe (Figure 3.3a). Nile red is widely employed for determining CAC values of amphiphiles, lipids, and polymers, owing to its strong sensitivity to local environment polarity.^[18] In aqueous solution, the dye is essentially non-emissive, but its fluorescence intensity rises sharply upon partitioning into hydrophobic domains, making it an ideal marker for amphiphilic self-assembly. Among the fuels tested, the CAC values decreased systematically with increasing alkyl chain length (Figure S3.2-S3.3). This trend strongly correlates with their calculated logP values, which increase from 0.27 (AP-C₈) to 3.30 (AP-C₁₄), reflecting the increased hydrophobicity of the longer tail fuels. The combined data clearly demonstrate that increasing tail length enhances the hydrophobic driving force, lowering the CAC and facilitating more efficient self-assembly. This correlation between CAC and logP underscores the central role of hydrophobicity in dictating aggregation behaviour in aqueous medium, and it further supports the kinetic observations where longer tails promoted faster imine formation.

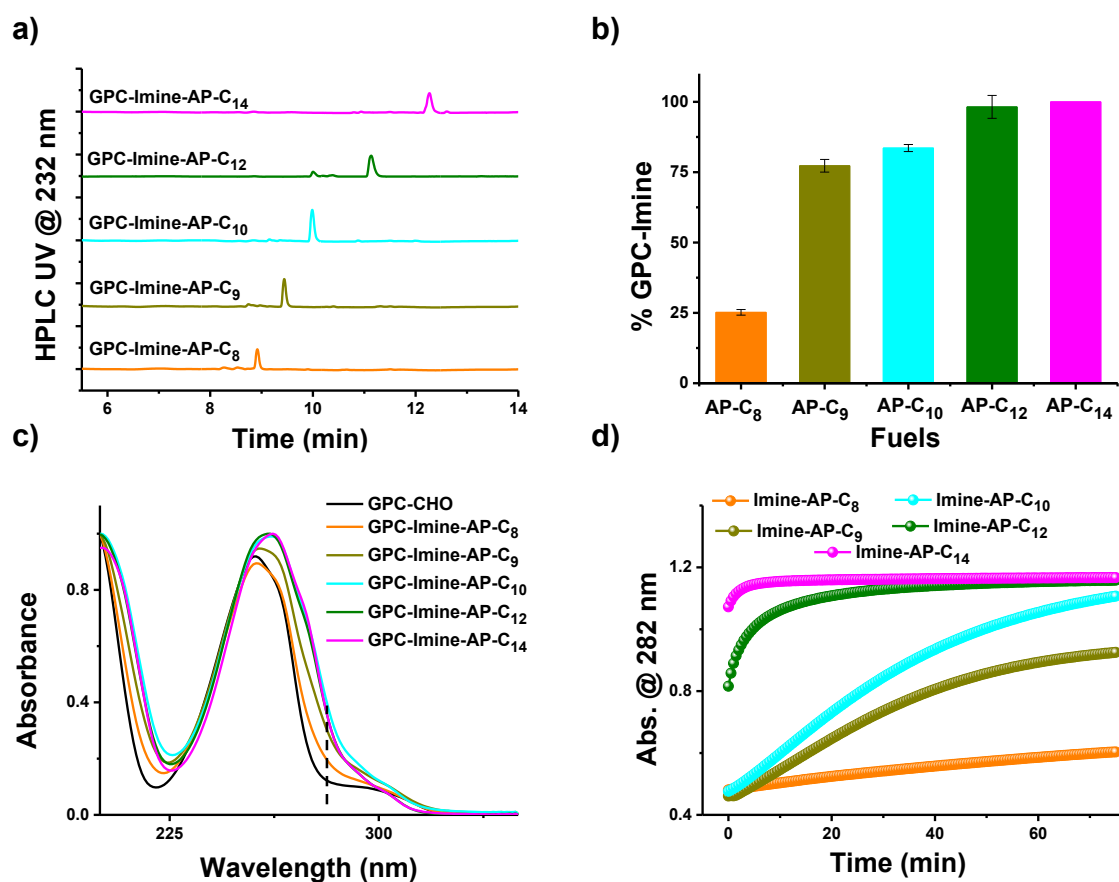


Figure 3.2. a) HPLC analysis of GPC-Imine formation after equilibrating the sample for 2h. b) Histogram showing percentage formation of GPC-Imine calculated 2 hrs after adding each fuel. c) Normalized absorption spectra of GPC-CHO and GPC-Imines formed after different fuel addition, (dotted line represents $\lambda = 282$ nm; 0.1mm cuvette). d) UV-Vis absorbance changes at $\lambda = 282$ nm showing the anabolic formation of GPC-Imine (1 mm cuvette). Conditions: 1 mM GPC-CHO, 3 mM Fuel.

To further investigate the effect of the self-assembly propensity on the formation kinetics of GPC-Imine, we compared the imine formation reaction by 3 mM and 6 mM concentrations of the AP-C₁₀ (Figure S3.4). The higher concentration of AP-C₁₀ fuel resulted in significantly faster formation of GPC-Imine-C₁₀ when compared to the lower concentration of fuel. This is because higher concentration increases the intermolecular interaction, i.e., higher self-assembly propensity. This leads to a greater decrease in free energy at higher concentrations, which enhances the thermodynamic driving force for organization. These findings suggest that the enhanced kinetics at higher concentrations are driven by the higher association constant of the resultant GPC-Imine.

Next, we investigated the supramolecular transformations that occur during GPC-Imine formation using fuels AP-C₁₀, AP-C₁₂, and AP-C₁₄, which displayed rapid formation kinetics, reaching ≥ 75 –100% conversion within 1 hour. DLS revealed distinct self-assembly behaviour depending on the

chain length (Figure 3.3b). For GPC-Imine-AP-C₁₀ and GPC-Imine-AP-C₁₂, the hydrodynamic size distributions were monodisperse, while GPC-Imine-AP-C₁₄ exhibited a clear bimodal distribution, indicative of a polydisperse system. The additional population observed in this case is likely due to aggregation of unreacted AP-C₁₄, which is more prone to self-aggregation because of its higher hydrophobicity. The DLS correlation profiles corroborated these findings, with AP-C₁₀ and AP-C₁₂ showing sharp decay curves consistent with monodisperse populations, while AP-C₁₄ displayed a broader decay profile characteristic of polydispersity (Figure 3.3c). These results establish that both hydrophobicity and chain length of the fuels strongly dictate the uniformity and size of the resulting supramolecular assemblies.

To further examine the morphology of these imine formation, confocal fluorescence microscopy was performed after labelling the assemblies with Nile red (Figure 3.3d–f). In line with DLS results, GPC-Imine-AP-C₁₀ showed well-defined, spherical vesicles with diameters of ~2 μm , although confocal spatial resolution limits mean only the larger vesicles are observable. The GPC-Imine-AP-C₁₂ system similarly produced stable, spherical vesicles with clear boundaries, again supporting the formation of monodisperse vesicular assemblies. In contrast, the GPC-Imine-AP-C₁₄ system resulted in less uniform vesicular structures, with evidence of irregular or polydisperse morphologies, consistent with its bimodal DLS distribution. Collectively, these confocal observations confirm the formation of vesicular assemblies across all three systems but also highlight the increasing tendency of longer chains to self-aggregate, thereby reducing structural uniformity. Together, DLS and fluorescence microscopy studies underscore the pivotal role of alkyl chain length in dictating supramolecular organization of GPC-Imine assemblies. These results provide mechanistic insights into how subtle changes in tail length tune in situ vesicle formation from simple precursors.

To explore the GPC-Imine assemblies under OOE conditions, we evaluated both their anabolic formation and catabolic degradation in situ, in the presence of lipase. Since we had already studied the metabolic synthesis using AP-C₁₂ (in chapter 2, Figure 2.4a) here we focused on GPC-Imine-AP-C₁₀ and GPC-Imine-AP-C₁₄. The enzyme concentration was systematically varied to assess its impact on vesicle disintegration kinetics. A clear dependence of enzyme concentration on the catabolic reaction was observed, higher concentrations of lipase accelerated the degradation kinetics of GPC-Imine, whereas lower concentrations allowed for prolonged vesicle lifetimes. The kinetic behaviour differed between GPC-Imine-AP-C₁₀ and GPC-Imine-AP-C₁₄. GPC-Imine-AP-C₁₀ demonstrated a clean degradation profile, with fast formation and slow, controlled disintegration within the specific time frame (Figure 3.4a). In contrast, GPC-Imine-AP-C₁₄ displayed a faster kinetics with significant tailing (Figure 3.4b). Kinetic analysis provided additional insights into the interplay between enzyme concentration and vesicle lifetime.

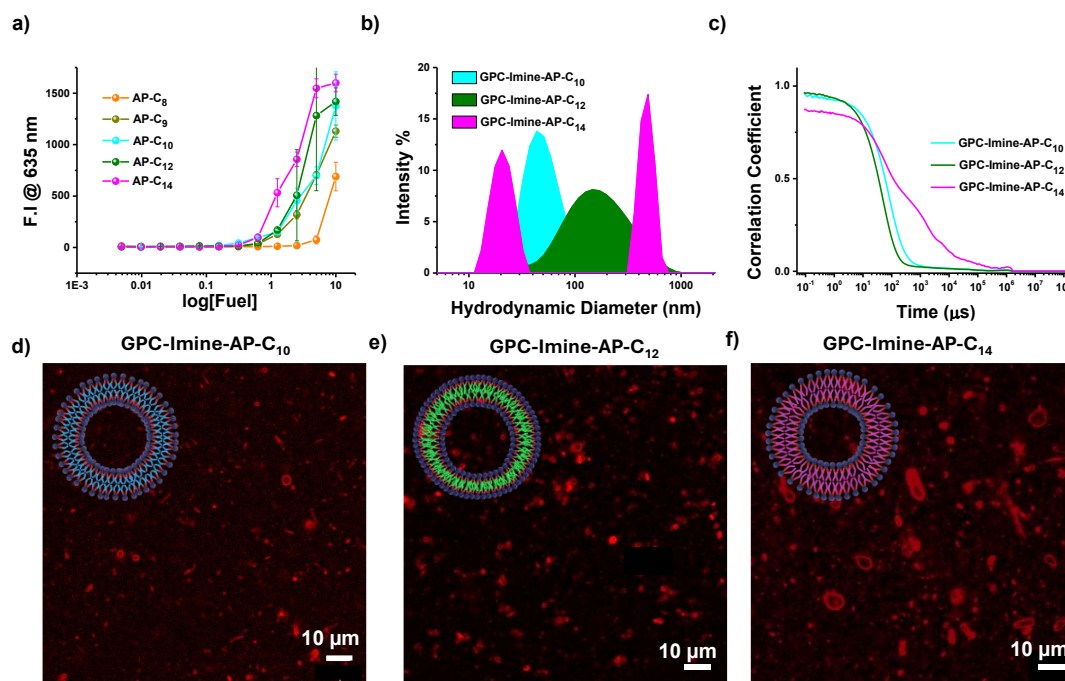


Figure 3.3. a) CAC of each fuel measured using Nile red probe. b) DLS data showing the hydrodynamic diameter of GPC-Imines nanostructures and c) shows the corresponding DLS correlation coefficient profile. d) Confocal microscopy images of vesicles formed from GPC-Imine-AP-C₁₀, e) GPC-Imine-AP-C₁₂ and f) GPC-Imine-AP-C₁₄. Conditions: 5mM GPC-CHO:15mM AP-C₁₀/C₁₄.

At a lipase concentration of 300 U/mL, GPC-Imine assemblies formed rapidly (10 minutes for AP-C₁₀ and 5 minutes for AP-C₁₄) and degraded more slowly, extending the vesicle lifetime. Conversely, at 600 U/mL, the systems exhibited faster degradation, leading to shorter lifetimes.

We next examined the reactivation potential of GPC-Imine assemblies by introducing fresh fuel after the initial degradation (Figure 3.4c-d). Such refueling mimics the operation of molecular machines and synthetic biological systems that require repeated energy input to sustain function. GPC-Imine-AP-C₁₀ displayed efficient regeneration upon the first refuel, rapidly reforming vesicles. The increase in absorbance during this cycle reflects enhanced formation, likely facilitated by the more hydrophobic environment established in the first round. However, the second refueling cycle exhibited damping, due to the accumulation of waste products from the catabolic process. GPC-Imine-AP-C₁₄ also showed regeneration of imines after the first cycle, with limited recovery upon refueling, reflecting its higher aggregation propensity and hydrophobic byproduct buildup. These results highlight how alkyl chain length dictates reactivation efficiency, with shorter chains enabling more robust regenerative cycles. The observed differences between GPC-Imine-AP-C₁₀ and GPC-Imine-AP-C₁₄ highlight the critical role of hydrophobicity and chain length in regulating both the stability and regenerative capacity of self-assembled systems. While shorter alkyl chains enable more stable and reactivatable systems, longer chains introduce challenges due to aggregation and hydrophobic waste accumulation.

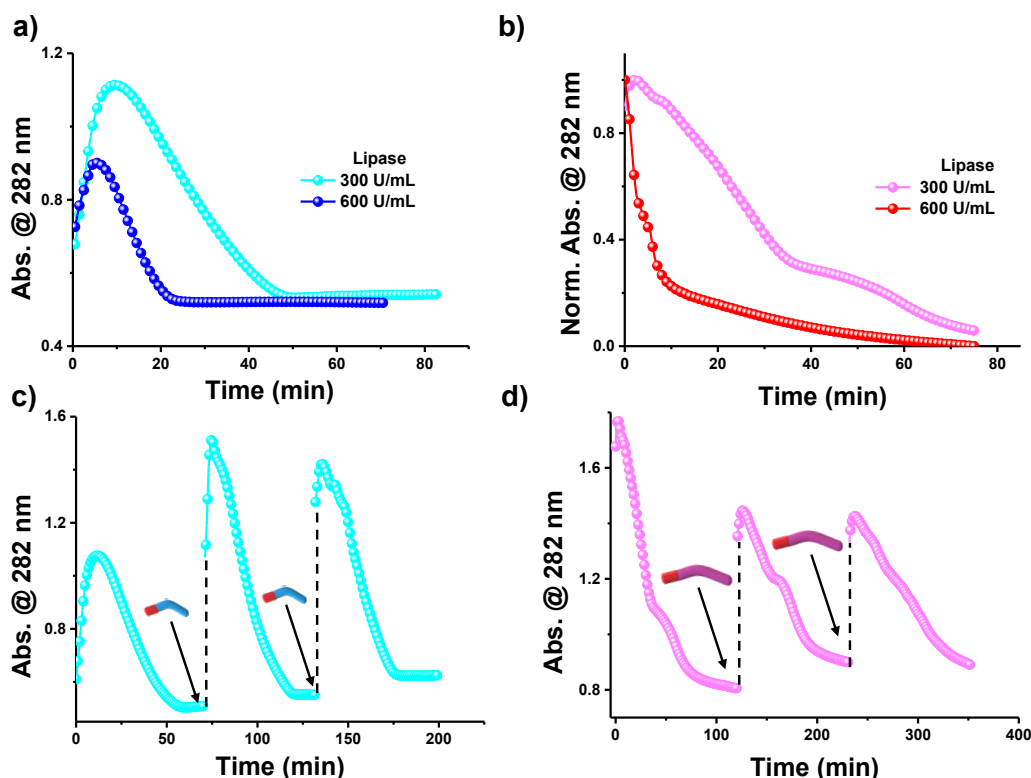


Figure 3.4. Time-dependent absorbance changes in the presence of lipase demonstrating the autonomous formation and degradation of a) GPC-Imine-AP-C₁₀ and b) GPC-Imine-AP-C₁₄, resulting in transient lipid vesicles. The unusual behaviour observed in the absorbance spectra can be attributed to the sedimentation of C₁₄ fatty acid precipitates generated as waste during hydrolysis (similar observation in chapter 2, Figure S2.36) c) Refueling the system with fresh batch of AP-C₁₀ fuel after first cycle with 1 mM GPC-CHO, 3 mM AP-C₁₀, 300 U/mL lipase d). Refueling the system with fresh batch of AP-C₁₄ fuel after first cycle with 1 mM GPC-CHO, 3 mM AP-C₁₄, 300 U/mL lipase.

Furthermore, we have shown that these chemically fueled vesicles can encapsulate and release the hydrophobic dye Nile red, with release kinetics governed by enzymatic activity and vesicle integrity (Figure S3.5). In the absence of lipase, both GPC-Imine vesicles remained in an equilibrium state, with no observable change in fluorescence, confirming their impermeable nature. Increasing the lipase concentration from 400 U/mL to 1000 U/mL resulted in a significant decrease in fluorescence intensity in both GPC-Imine-AP-C₁₀ and GPC-Imine-AP-C₁₄, indicating that higher enzymatic activity accelerates hydrolysis and facilitates cargo release. The decrease in fluorescence corresponds to the release of Nile red into solution, highlighting the role of lipase in modulating release dynamics and its potential relevance for drug delivery applications. Notably, GPC-Imine-AP-C₁₀ displayed a smooth and gradual fluorescence decay, suggesting that its chain length provides a balance between vesicle stability and susceptibility to enzymatic degradation, leading to a predictable release profile. By contrast, GPC-Imine-AP-C₁₄ showed an inconsistent release

pattern across enzyme concentrations, implying that its longer chain length may introduce structural heterogeneity or kinetic barriers that complicate controlled cargo release.

In natural systems, multiple fuels often coexist and compete for metabolic pathways, and this competition can bias reaction outcomes. Specifically, lipid biosynthesis rarely relies on a single precursor but instead draws from a pool of activated fatty acids donors operating concurrently. Hence, we studied the phospholipid formation in the presence of AP-C₁₀ and AP-C₁₂ fuels to probe how competition controls lipid formation and assembly. When we added both the fuels in a ratio of 2:2 with GPC-CHO at equilibrium conditions in the absence of lipase, the in situ formation of phospholipids generated molecularly asymmetric lipids with mixed acyl chain lengths (Figure 3.5a). Using HPLC we found that the percent of GPC-Imine with both tail AP-C₁₀ was 13%, GPC-Imine with mixed tail composition (one tail AP-C₁₀ and another one AP-C₁₂) was 45 % and the one with both AP-C₁₂ tail was 42% (Figure 3.5b-c). Under equilibrium conditions, the distribution of lipid species reflects the relative availability of precursors and the thermodynamic stability of the products. As expected, when only a single fuel was supplied, the system exclusively produced the corresponding symmetric lipids. The fraction of these mixed tail lipids is tunable by adjusting the concentration of the short-chain precursor, reaching up to 45% under optimized conditions (Table S3.1). These observations highlight a fundamental feature of equilibrium chemistry in the absence of continuous fueling or external driving forces, lipid formation relaxes into a mixed population with no persistent selectivity.

Although we observed that supplying two fuels simultaneously drives the system towards different lipid populations, we next asked whether lipid assemblies can undergo compositional exchange after their initial formation of vesicles from one fuel and then introducing the other (Figure 3.5d). We added AP-C₁₂ fuel to the preformed lipid vesicles of GPC-Imine-AP-C₁₀. The initial population of 100 % GPC-Imine-AP-C₁₀ imine shifted markedly to ~11% along with ~49% GPC-Imine-AP-C₁₂ and ~40% mixed tail GPC-Imine-AP-C₁₂/C₁₀ imine (Figure 3.5d-e). In the reverse experiment, preformed GPC-Imine-AP-C₁₂ vesicles supplied with AP-C₁₀ fuel produced ~25% GPC-Imine-AP-C₁₀, ~45% mixed, and ~30% GPC-Imine-AP-C₁₂ lipids (Table S3.2). Compared to the first case, the change in composition of lipids was less extreme, with a larger fraction of GPC-Imine-AP-C₁₂ imine remaining. Finally, when preformed vesicles of both AP-C₁₀ and AP-C₁₂ were mixed and allowed to remodel, the system relaxed to ~20% GPC-Imine-AP-C₁₀, ~46% mixed, and ~35% GPC-Imine-AP-C₁₂ lipids, with the mixed tail imines enriched (Figure 3.5e). These experiments demonstrate that even under equilibrium conditions, our GPC-Imine phospholipid vesicles are not compositionally frozen but can undergo dynamic reorganization when multiple precursors are present or when vesicle populations are mixed. Such spontaneous redistribution offers a conceptual analogue to the lipid exchange and remodeling seen in natural membranes, albeit in the absence of energy-dissipating pathways.

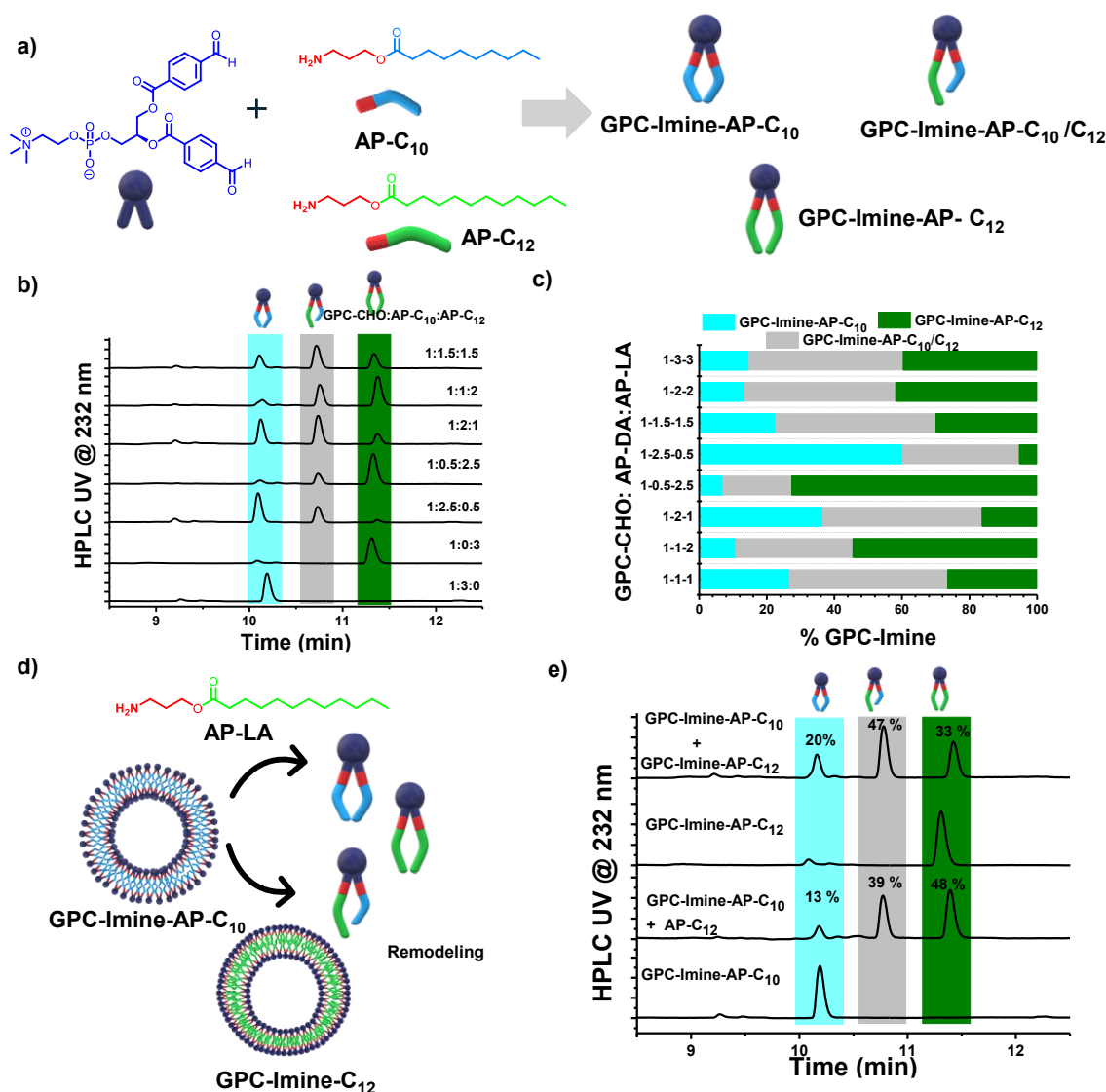


Figure 3.5. a) Schematic representation of GPC-CHO reacting with two fuels to generate homo- and mixed lipid populations. b) HPLC traces of GPC-Imine formation at different molar ratios of AP-C₁₀ and AP-C₁₂. c) Percentage distribution of the resulting GPC-Imine species. d) Schematic of fuel addition to preformed GPC-Imine vesicles. e) Representative HPLC chromatograms showing the corresponding lipid populations.

Importantly, the extent of remodeling depends on the order of precursor addition where GPC-Imine-AP-C₁₀ vesicles were almost completely remodelled by the fuel AP-C₁₂, whereas GPC-Imine-AP-C₁₂ vesicles retained a larger fraction of their original population. This difference suggests that GPC-Imine-AP-C₁₀ vesicles are more dynamic, likely due to weaker hydrophobic stabilization and faster exchange kinetics, while GPC-Imine-AP-C₁₂ vesicles are more stable and compositionally persistent. The consistent enrichment of mixed tail lipids across all experiments highlights their thermodynamic accessibility once exchange occurs. Altogether, these results show that our lipid

vesicles exhibit a form of membrane plasticity, reminiscent of how biological membranes remodel their composition in response to changing environments.

Confocal fluorescence imaging provided direct insight into the morphological consequences of lipid composition. In system with high mixed tail lipids composition, the dominant structures were rugby ball like vesicles (Figure 3.6a-b, Figure S3.6). To confirm that these remodeled structures are vesicular rather than random aggregates, we encapsulated the water-soluble dye HPTS during their formation. Confocal fluorescence microscopy revealed well-defined elongated compartments with the dye localized in their interior, unequivocally demonstrating the presence of an aqueous lumen (Figure S3.7). This indicates that the asymmetry in lipid tails introduced packing frustration leading to altered membrane packing and curvature stress, giving anisotropic vesicular structures.^[19-22] In contrast, systems containing a higher proportion of symmetric (homo-tail) lipids exhibited well-defined spherical vesicles as the predominant population (Figure 3.6c). This structural variation highlights how molecular-level asymmetry and hydrophobic mismatch in lipid tails can be translated into mesoscale curvature effects, providing direct visual evidence of lipid remodeling.^[23]

To further explore the equilibrium behavior of multi-fuel systems, we investigated mixtures of AP-C₁₀, AP-C₁₂, and AP-C₁₄ in different combinations (Figure S3.8a, Table S3.1). When AP-C₁₂ and AP-C₁₄ were combined, the system consistently evolved to a mixed-tail population, with significant fractions of homo-lipid population (Figure S3.8b). A similar outcome was observed for AP-C₁₀ and AP-C₁₄ mixtures, where the mixed population dominated over the respective homo-lipids (Figure S3.8c). Extending this approach to a ternary mixture of all the three fuels, the distribution converged to mixed populations, with all three fuels contributing to the formation of asymmetric lipids (Figure S3.9a). Furthermore, introducing two fuels to preformed vesicular assemblies also drove the system toward mixed-tail lipid species, closely mirroring the compositional trends observed under direct equilibrium mixing (Figure S3.9b, Table S3.2). These observations highlight that under equilibrium conditions, the system naturally favors statistical mixing of tails, resulting in heterogeneous lipid populations rather than maintaining distinct homo-lipid compartments.

Confocal imaging across all systems confirmed the predominant formation of phospholipid vesicles (Figure S3.10). Interestingly, only the combination of AP-C₁₀ and AP-C₁₂ with GPC-CHO gave rise to unusual rugby-ball like assemblies instead of classical vesicles. This observation motivated us to test whether such aberrant morphologies could be remodeled by introducing an additional fuel (Figure 3.6d). Confocal imaging revealed that the addition of AP-C₁₄ to the pre-formed system transformed rugby-ball structures into well-defined vesicles (Figure 3.6e). This provides compelling evidence that fuel addition can reconfigure metastable supramolecular structures into

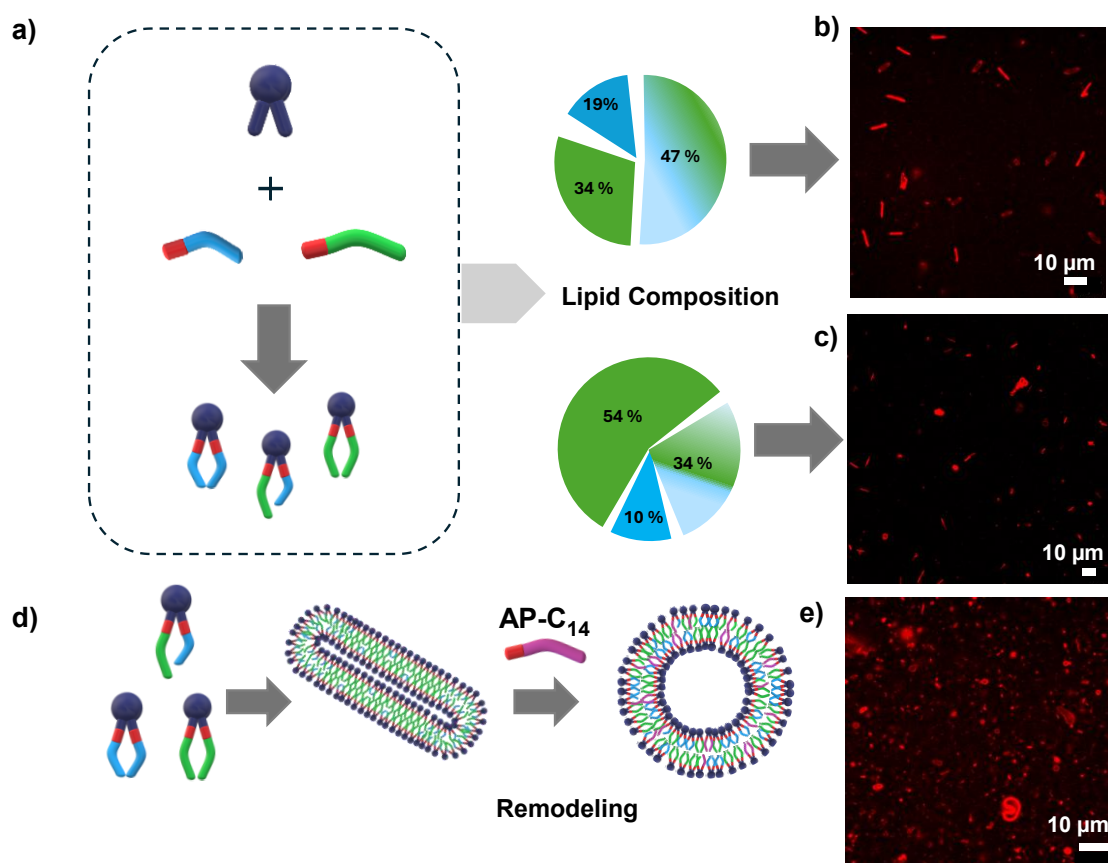


Figure 3.6. a) Schematic showing the addition of AP-C₁₀ and AP-C₁₂ to GPC-CHO, yielding different lipid compositions and corresponding confocal images; b) systems enriched in mixed-tail species predominantly form rugby ball-like vesicles, whereas c) homo-lipid populations favour spherical vesicles. d) Schematic representation of the in situ transformation of rugby ball-like vesicles into spherical vesicles upon introduction of an additional fuel. e) Confocal image of vesicles obtained after addition of AP-C₁₄.

vesicles, underscoring the capacity of our system to undergo in situ structural remodeling in response to new chemical inputs through dynamic imine bonds.

Having established the equilibrium assembly profiles, we next asked whether OOE control in presence of lipase enzyme could bias the lipid composition in a manner similar to natural membranes. We first examined the addition of AP-C₁₀ and AP-C₁₂ fuels to GPC-CHO in the presence of lipase. Time-dependent HPLC studies showed very little change in composition of homo- and mixed lipid species over the period when compared to the same system at equilibrium conditions (Figure 3.7a-c). This suggests that the enzyme could not discriminate between the two tails due to their similar hydrophobicity and nearly identical deactivation kinetics. Many lipases exhibit limited discrimination between medium, closely spaced chain lengths (C₁₀ vs C₁₂).^[24] Since the hydrolysis rates for GPC-Imine-AP-C₁₀ and GPC-Imine-AP-C₁₂ lipids are almost similar, the enzyme hydrolyses both at comparable rates. The simultaneous re-formation of imines from both tails then yields no net change in lipid composition. Nevertheless, in this system we did not observe

complete degradation of the constituent lipids, unlike in the case of individual vesicles. This is likely due to the restricted binding and reduced catalytic efficiency of lipase on the low-curvature rugby-ball structures, which limit enzymatic access to the membrane surface.

To overcome this issue, we mixed the AP-C₁₂ fuel with a shorter AP-C₈ fuel and introduced to the GPC-CHO to deliberately create kinetic asymmetry and hydrophobic contrast among the two fuels (Figure 3.7d, Figure S3.11a). As anticipated, the lipid species formed from these fuels revealed preferential remodeling, which was not seen in the AP-C₁₀/AP-C₁₂ system. Under equilibrium conditions, GPC-Imine-AP-C₈ lipids represented only a minor fraction ($\approx 7\%$) (Figure 3.7e-f). By contrast, under OOE conditions, the system showed a dramatic enrichment of this species, rising to nearly $\approx 25\%$ (Figure 3.7g). Similarly, the mixed lipid population also increased to 52% from equilibrium amount of 42%. This selective amplification was coupled to a rapid depletion of the homo- GPC-Imine-AP-C₁₂ lipid species. Through subsequent re-equilibration via imine exchange, the system redistributed the remaining lipids, leading to enrichment of both the mixed and the shorter lipid homo- populations. These results reveal that kinetic asymmetry, in this case stemming from hydrophobic tail length, can be utilized by an enzymatic cycle to drive lipid composition away from the thermodynamic distribution (Figure 3.7h). We observed an almost complete degradation of the assemblies by lipase, due to the formation of higher curvature vesicular structures (Figure S3.11b). The increased curvature of vesicles likely enhances enzyme accessibility to the membrane surface, in contrast to low-curvature rugby-ball structures hindering catalytic activity. This behaviour is strikingly reminiscent of natural membrane remodeling, where enzymatic turnover and metabolic flux bias lipid populations toward non-equilibrium states essential for function. Our findings suggest that even simple amphiphilic systems, when coupled to catalytic cycles, can acquire adaptive traits usually associated with biological membranes.

3.3 Conclusion

We have demonstrated the chemical fuel-driven formation and degradation of phospholipids using fuels with varying chain lengths, highlighting the interplay between molecular structure, hydrophobicity, and kinetics during self-assembly. We found that the kinetics of imine formation is strongly influenced by hydrophobicity where longer, more hydrophobic tails promote faster lipid formation, while shorter tails assemble slowly. Crucially, when systems are driven under OOE conditions, these kinetic differences dictate compositional outcomes in ways inaccessible at equilibrium conditions. While equilibrium conditions always yielded mixed asymmetric species, driving the system OOE revealed kinetic asymmetries that biased membrane composition toward distinct lipid populations. These results provide deeper insights into the interplay between molecular asymmetry and curvature generation in membranes, offering a practical model to study shape regulation in both synthetic and biological contexts. Furthermore, it underscores how fuel-

driven self-assembly can serve as a simple yet powerful route to control vesicle architecture, with structural features dictated by the balance between homo- and mixed-tail lipid populations. By linking hydrophobicity, kinetics, and composition, these results establish a framework for how synthetic systems can be programmed to undergo lipid remodeling under dissipative conditions, bringing us closer to constructing artificial membranes that not only compartmentalize but also evolve dynamically, as living membranes do.

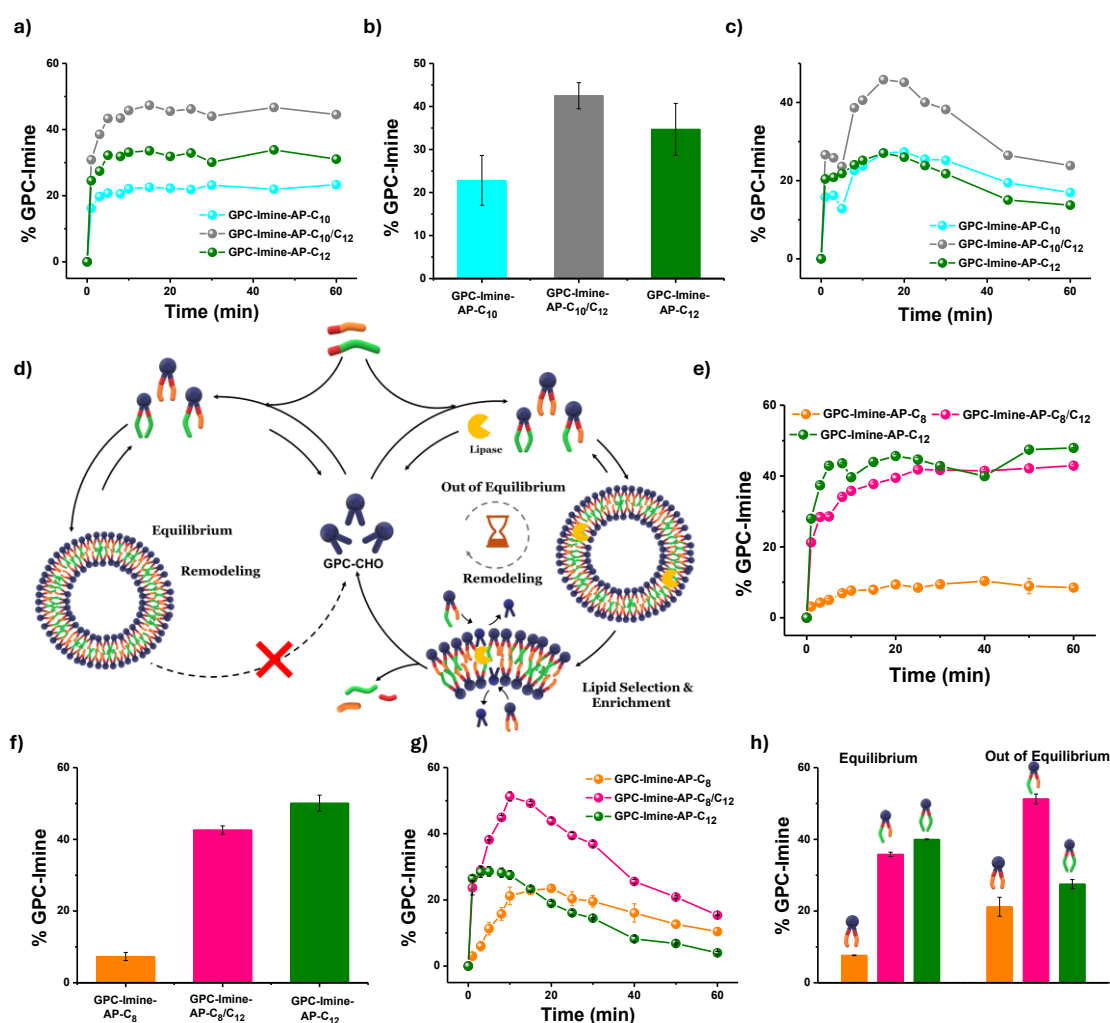


Figure 3.7. a) Kinetics of formation of various GPC-Imine lipid populations from AP-C₁₀ and AP-C₁₂ fuels at equilibrium obtained from HPLC peak area. b) Percentage of lipid populations after 1h (equilibrium). c) Kinetics of formation and degradation of lipid populations from AP-C₁₀ and AP-C₁₂ fuels under OOE conditions obtained from HPLC peak area. d) Schematic showing the equilibrium and OOE formation of lipids with AP-C₈ and AP-C₁₂ fuels. e) Kinetics of formation of various GPC-Imine lipid populations from AP-C₈ and AP-C₁₂ fuels at equilibrium conditions f) Percentage of lipid populations after 1h (equilibrium). g) OOE kinetics of lipid populations from AP-C₈ and AP-C₁₂ fuels in presence of lipase. h) Histogram showing the changes of population at equilibrium and OOE conditions at time, t=10 minutes. Conditions: 1mM GPC-CHO, 2:2 Fuels and enzyme 600U/mL in OOE conditions

Although we could not achieve a completely pure selection or enrichment of a single lipid population under OOE conditions, our results clearly show that when sufficient kinetic or structural differences exist, selective remodeling can occur. To our knowledge, this is the first example of such behaviour in a synthetic phospholipid system. At the same time, the approach has limitations such as the incomplete selection, and the outcome is influenced by the factors such as curvature, tail-length mismatch, and enzyme accessibility. Nevertheless, this study provides the first proof of synthetic OOE phospholipid vesicles that are capable of selective remodeling, offering a minimal model of membrane plasticity. These findings lay the foundation for engineering adaptive, life-like compartments for synthetic biology and provide insights into how primitive membranes may have achieved dynamic remodeling through simple chemical principles.

3.4 Materials and Methods

3.4.1 Materials

Octanoic acid, Decanoic acid, Tetradecanoic acid, Nile Red dye and Lipase (*Candida Rugosa*) were purchased from Sigma Aldrich. HEPES sodium salt, n- Nonanoic acid and Formic acid were purchased from Fisher Thermos Scientific. Sodium Cyanoborohydride (NaBH_3CN) was purchased from Alfa Aesar. Chloroform, Diethyl Ether, Methanol, anhydrous Dichloromethane (DCM), anhydrous Dimethylformamide (DMF) were purchased from Sigma Aldrich and used without any further purification. All the NMR solvents were purchased from Sigma Aldrich.

3.4.2 Methods

3.4.2.1 Column Chromatography

Column chromatography was performed on silica gel 60. Analytical TLC was performed on Merck Kieselgel 60 with appropriate mixtures of solvents as eluents. Products were detected by using standard staining agents like Iodine, KMnO_4 solution followed by heating, Ninhydrin stain and UV visualization.

3.4.2.2 NMR Measurements

NMR spectra were recorded with a Bruker 400 MHz Avance III NMR spectrometer with chemical shifts reported in parts per million (ppm). Splitting patterns are designated as s, singlet; d, doublet; bs, broad singlet; m, multiplet; t, triplet; q, quartet; quin, quintet and br, broad.

3.4.2.3 UV-Vis Absorption spectroscopy

Electronic absorption spectra were recorded with an Agilent Cary 5000 spectrometer at scanning speed of 200 nm per min from 200-700 nm using 1 mm and .1 mm quartz cuvette. For the kinetic

experiments, the instrument was set to take measurements at an interval of 30 seconds for a period of 2 hours at 282 nm wavelength.

3.4.2.4 Confocal Imaging

For fluorescent labelling of vesicles, a 5 mM stock solution of Nile red in acetone was first prepared. Then the required volume was transferred to an eppendorf tube, and the solvent was evaporated to dryness. To the resulting thin film of Nile red, the aldehyde and fuel were added, and the mixture was allowed to react and form GPC-Imine vesicles, ensuring incorporation of the dye into the lipid structures. The tube was gently rotated on a rotor for 2–3 hours at room temperature to ensure proper incorporation of Nile red into the lipid structures. After incubation, 20 μ L of the sample solution was added to an 8 well ibidi μ -slide and imaged. Confocal microscopy was performed using a Zeiss LSM 800 system equipped with a 63x/1.40 glycerol-immersion objective and a PMT detector. Image cropping, contrast, and brightness adjustments were performed using Fiji-ImageJ software.

3.4.2.5 HPLC-MS

The imine formation was quantified using an Agilent HPLC-MS instrument fitted with Poroshell 120 C18 analytical column. A 10 μ L of sample was reduced using an excess of sodium cyanoborohydride (NaBH_3CN , 1mg in ethanol) and diluted to 800 μ L using acetonitrile: water (50:50) with 0.1% formic acid. The HPLC analysis was performed with an injection volume of 20 μ L and a constant flow rate of 0.400 mL/min. The mobile phase consisted of Solvent A (water with 0.1% formic acid) and Solvent B (acetonitrile with 0.1% formic acid). The gradient elution program was as follows: initially, 90% Solvent A and 10% Solvent B (0 minutes); transitioning to 70% Solvent A and 30% Solvent B at 2 minutes; reaching 10% Solvent A and 90% Solvent B at 9 minutes and maintained until 15 minutes; followed by a re-equilibration to the initial condition of 90% Solvent A and 10% Solvent B at 16 minutes, which was maintained until 17 minutes. In all the cases, GPC-Imine formation was confirmed by monitoring the UV signal at 232 nm followed by performing the MS analysis of the same peak.

3.4.2.6 DLS

DLS measurements of GPC-Imine samples were performed in a Zetasizer Ultra (Malvern UK) instrument employing a 633 nm laser at a back scattering angle of 173° . GPC-Imine samples were prepared at (1mM GPC-CHO: 3mM fuel) concentrations and equilibrated for 2h. The hydrodynamic diameters were recorded to measure polydispersity followed by the measurement of coefficient correlation in samples.

3.4.2.7 CAC using Nile red

The Nile red probe method was utilized to determine the CAC's of the fuels. A stock solution of Nile red (5 mM) was prepared in ethanol, and a series of fuel solutions with concentrations ranging from 0 to 10 mM were prepared in triplicates in a 96-well plate. Nile red was added to each fuel at a constant concentration of 20 μ M. The plate was kept undisturbed in the dark for 16 hours to reach equilibrium. Fluorescence intensity was then measured at an emission wavelength of 635 nm using a Tecan Spark plate reader, with an excitation wavelength of 545 nm.

3.4.2.8 Nile red Localization and Release Studies

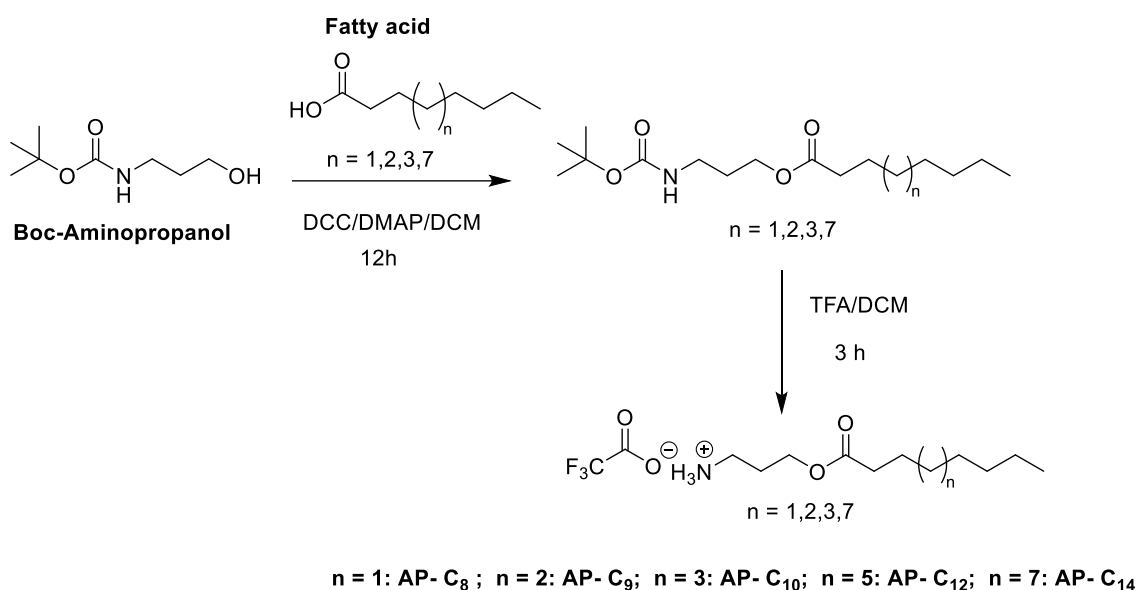
A 10 mM stock solution of Nile red was prepared in acetone. An aliquot of 5 μ L was transferred into a glass vial, and the solvent was removed under vacuum to yield a thin film of Nile red. Required amounts of GPC-CHO (1 mM) and fuel (3 mM) were then added to the vial and thoroughly mixed, allowing efficient localization of Nile red during vesicle formation. Kept the sample in a rotor for 2hrs for effective localization of Nile red. From this mixture, 200 μ L of the Nile Red -loaded GPC-Imine solution was added into a 96-well plate, and the initial fluorescence emission at 635 nm was recorded on a Tecan Spark Plate Reader using an excitation wavelength of 545 nm. Different concentrations of lipase enzyme were added and the changes in the fluorescence intensity over time was monitored at room temperature by using Tecan plate reader. Fluorescence values were normalized against the initial signal at 635 nm, which served as the baseline prior to enzyme addition.

3.4.2.9 Pyranine (HPTS) Encapsulation

Required amount of GPC-CHO (5mM), AP-C₁₀ (10 mM) and AP-C₁₂ (10 mM) were added to HEPES buffer containing 2 mM pyranine dye and allowed to self-assemble in situ. After equilibrating for 2 hours, 20 μ L of the sample is mixed with 2 mM of membrane impermeable quencher p-xylene-bis-pyridinium bromide (DPX) and directly imaged in an ibidi μ -slide using Zeiss LSM 800 microscope.

3.4.2.10 Synthetic Schemes

Synthesis of AP-C₈, AP-C₉, AP-C₁₀ and AP-C₁₂ is shown in Scheme S3.1.



Scheme S3.1: Synthesis of chemical fuels having different carbon chain length.

3.4.2.11 Synthesis of GPC-CHO and AP-C₁₂

GPC-CHO and AP-C₁₂ were prepared according to reported procedure of chapter 2.^[15]

3.4.2.12 Synthesis of N-Boc-Amino propanol Esters

All the Boc-protected fuels were synthesized through reported procedure.^[25]

3.4.2.13 Synthesis of Amino propanol Ester Fuels

Boc-Amino ester fuel (1 equiv.) was taken in a 100 mL round bottom flask dissolved in 4 ml of dichloromethane (DCM) and placed in an ice bath at 0 °C. Then TFA (20 equiv.) was added drop wise to the solution. After 15 minutes the ice bath was removed, and the reaction was stirred at room temperature for 3 hours. After 3 hours a thin layer chromatography (TLC) was performed to check the reaction was already completed. The solvent was removed by rotavapor and obtained the required amino ester fuel. The final product was kept under high vacuum overnight to remove the excess TFA.

Aminopropanol-Octanoate (AP-C₈): Transparent Oil

Yield: 850 mg (Quantitative Yield).

¹H NMR (400 MHz, DMSO-d₆) δ 7.83 (s, 3H), 4.08 (t, J = 6.3 Hz, 2H), 2.89-2.81 (m, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.89-1.82 (m, 2H), 1.55-1.47 (m, 2H), 1.25 (s, 8H), 0.87(m, 3H).

ESI-MS (positive mode): m/z calculated for C₁₁H₂₃NO₂ 201.17; found C₁₁H₂₄NO₂⁺ 202.1.

Aminopropanol-Nonanoate (AP-C₉): Yellow Oil

Yield: 411 mg (Quantitative Yield).

^1H NMR (400 MHz, DMSO- d_6) δ 7.80 (s, 3H), 4.08 (t, J = 5.9 Hz, 2H), 2.89 – 2.81 (m, 2H), 2.31 (dt, J = 7.4 Hz, 2H), 1.89 – 1.82 (m, 2H), 1.55 – 1.46 (m, 2H), 1.24 (s, 10H), 0.87 (m, 3H).

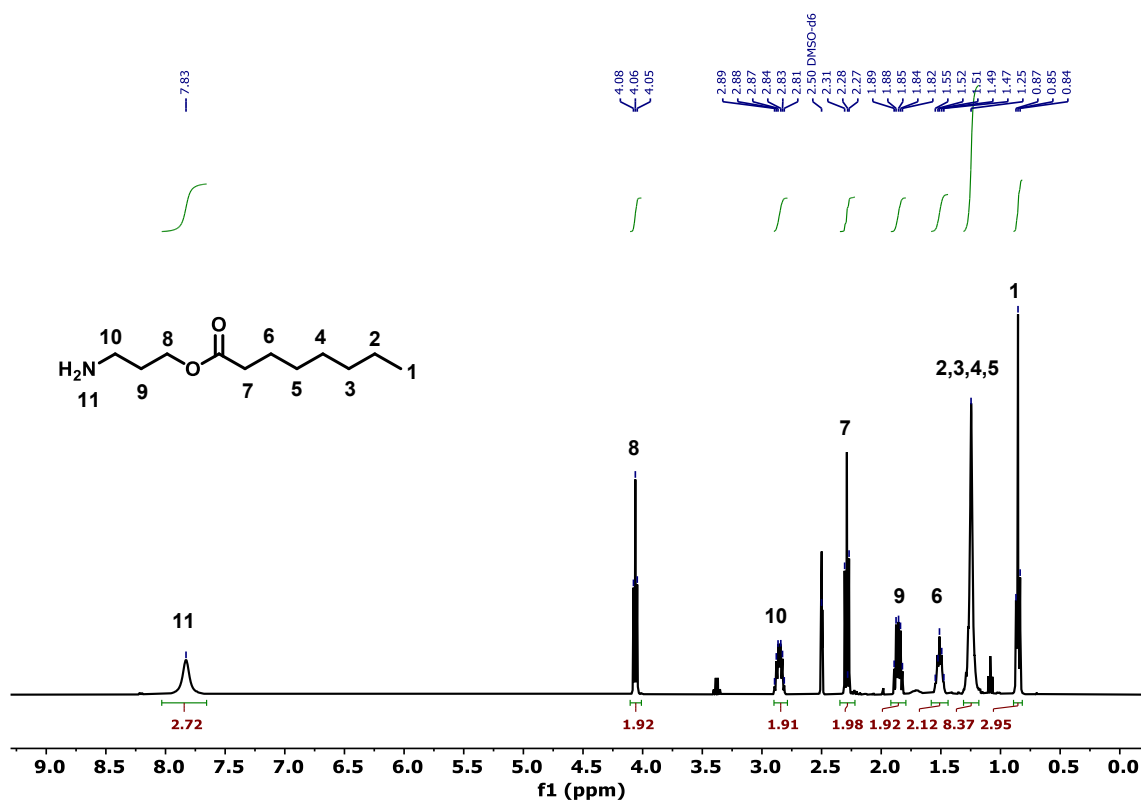
ESI-MS (positive mode): m/z calculated for $\text{C}_{12}\text{H}_{25}\text{NO}_2$ 215.19; found $\text{C}_{15}\text{H}_{26}\text{NO}_2^+$ 216.2.

Aminopropanol-Decanoate (AP- C_{10}): Transparent Oil

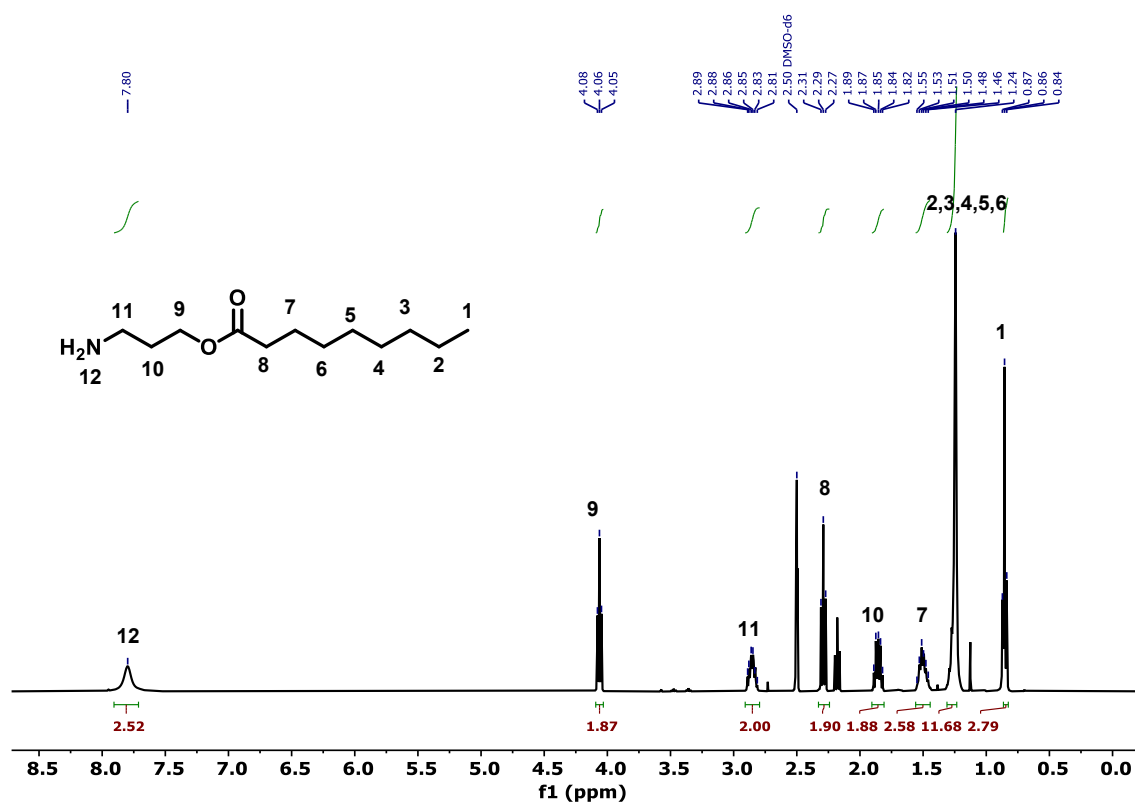
Yield: 826 mg (Quantitative Yield).

^1H NMR (400 MHz, DMSO- d_6) δ 7.79 (s, 3H), 4.08 (t, J = 6.3 Hz, 2H), 2.90-2.82 (m, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.91-1.82 (m, 2H), 1.55 – 1.50 (m, 2H), 1.25 (s, 13H), 0.88 (m, 3H).

ESI-MS (positive mode): m/z calculated for $\text{C}_{13}\text{H}_{27}\text{NO}_2$ 229.20; found $\text{C}_{13}\text{H}_{28}\text{NO}_2^+$ 230.2.



^1H NMR of AP- C_8



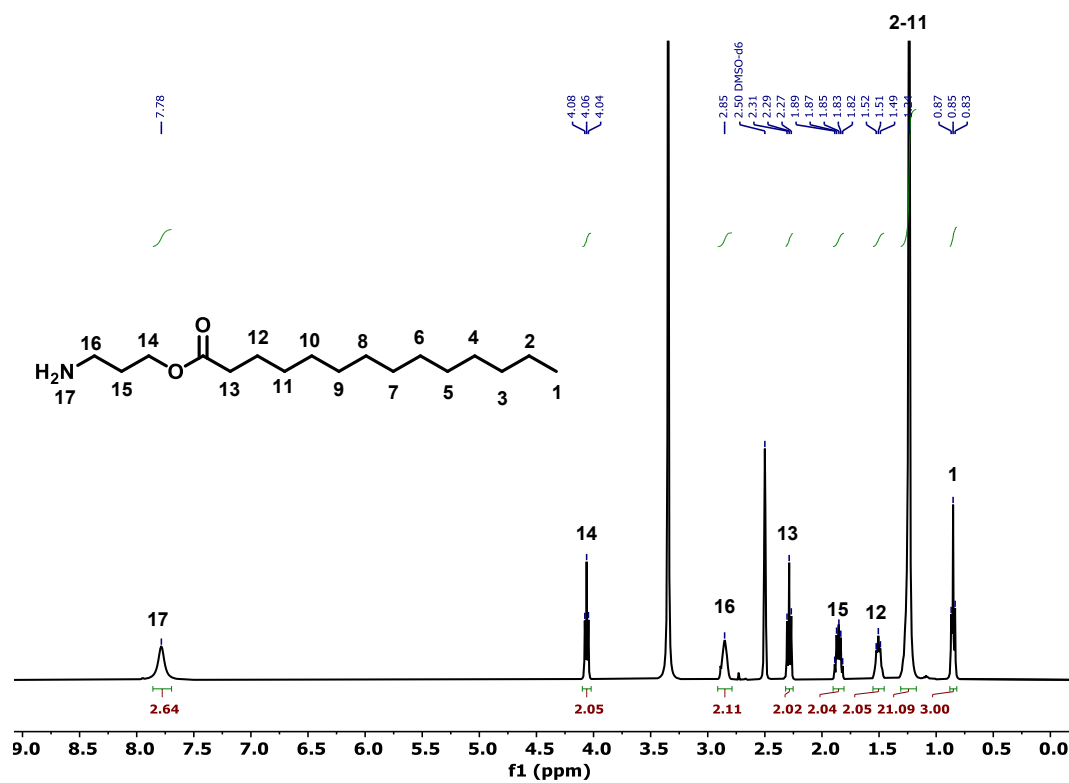
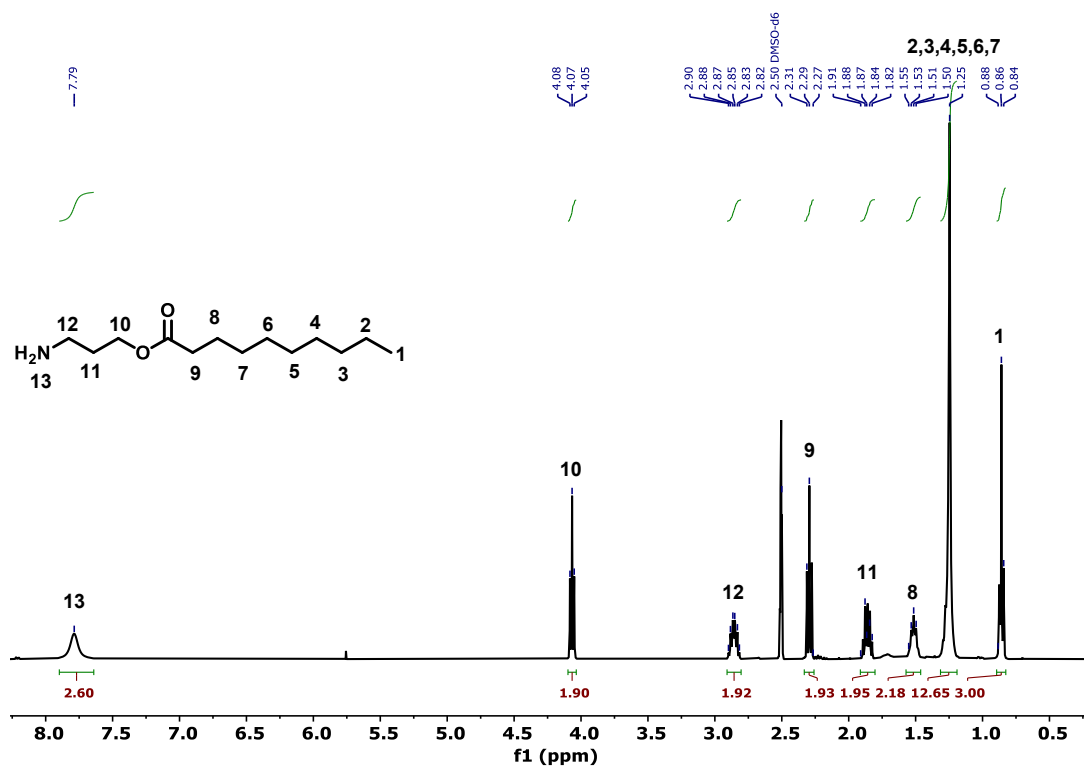
¹H NMR of AP-C₉

Aminopropanol-Myristate (AP-C₁₄): White flaky solid

Yield: 105.7 mg (10%). (*yield is low due to experimental error*)

¹H NMR (400 MHz, DMSO-d₆) δ 7.78 (s, 3H), 4.08 (t, $J = 6.2$ Hz, 2H), 2.85 (m, 2H), 2.31 (t, $J = 7.6$ Hz, 2H), 1.89-1.82 (m, 2H), 1.52 – 1.49 (m, 2H), 1.44 (s, 9H), 1.24 (s, 20H), 0.87 (m, 3H).

ESI-MS (positive mode): m/z calculated for C₁₇H₃₅NO₂ 285.27; found C₁₇H₃₆NO₂⁺ 286.3.



3.4.2.14 Sample Preparation

Stock solutions of GPC-CHO (50 mM), AP-C8, AP-C9, AP-C10 (50 mM each), and AP-C14 (20 mM) were prepared in a 100 mM HEPES buffer at pH 7.4. Working concentrations were then prepared from these stock solutions. The required amount of the appropriate fuel was injected into a vial containing GPC-CHO aldehyde in the buffer. For samples containing lipase, the required amounts of GPC-CHO and lipase were mixed in the buffer solution. Subsequently, the required fuel was added, and the entire solution was vortexed for 30 seconds.

For the competition experiments, mixed the required amount of fuels together separately and injected into the HEPES buffer containing GPC-CHO. Lipase was added before the addition of fuels in the OOE competition experiments.

3.5 Supporting Figures

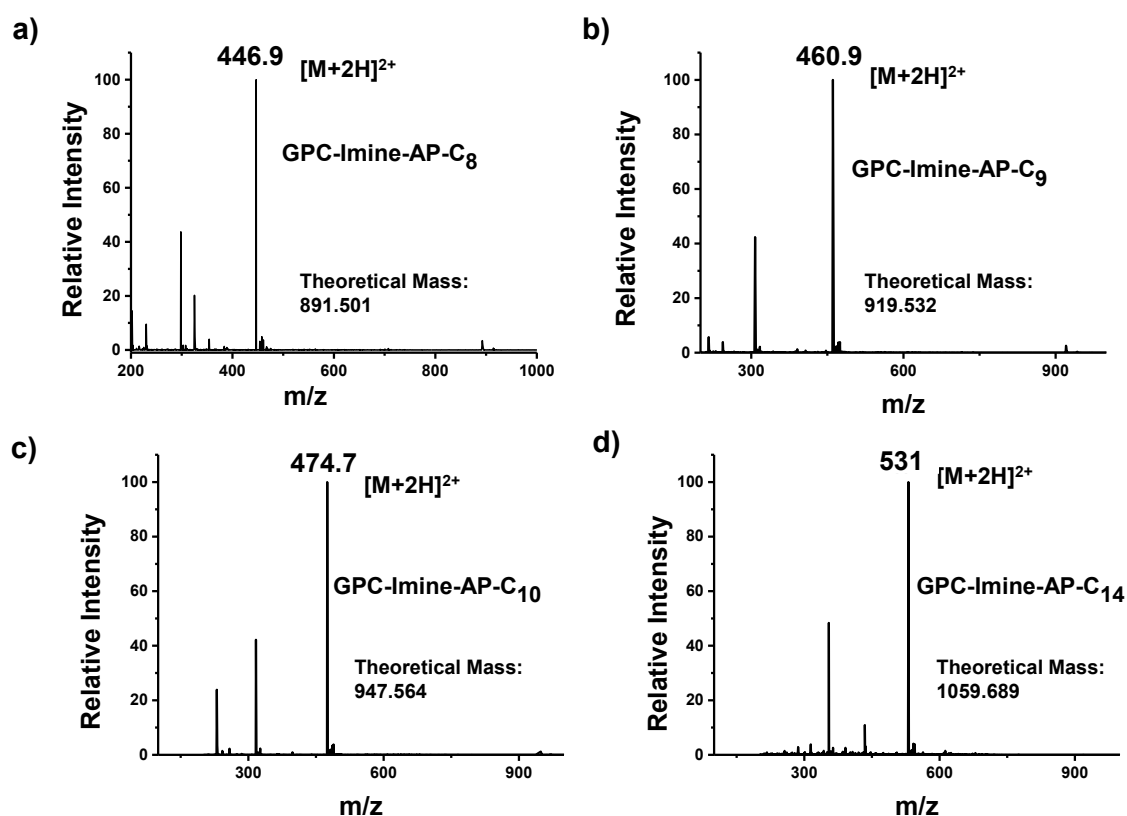


Figure S3.1. MS data showing the $[M+2H]^{2+}$ ion of the GPC-Imines formed from respective fuel.

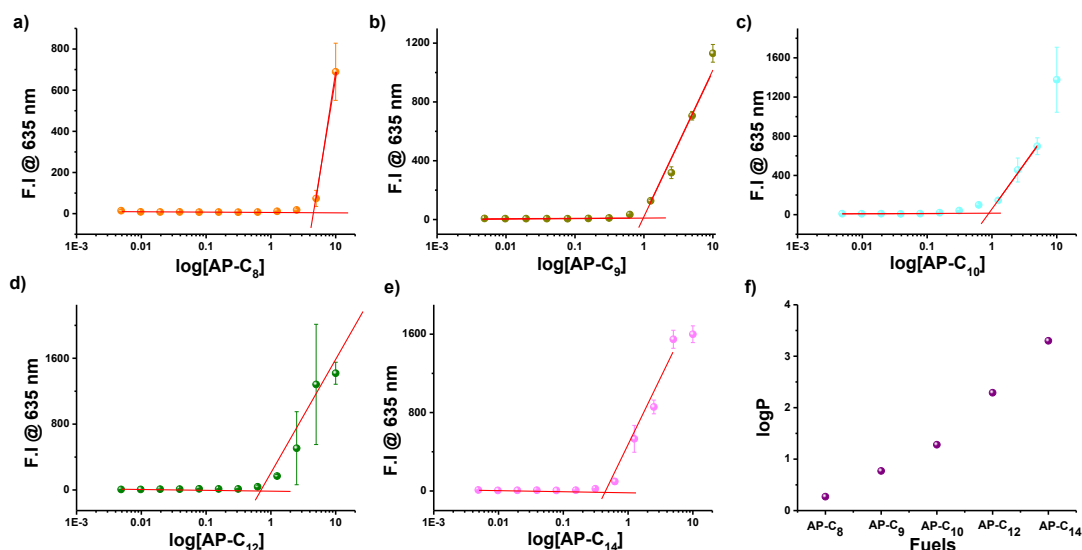


Figure S3.2: a)-e) CAC of amphiphiles using Nile red fluorescence. The intersection point plots corresponds to the CAC value. f) Plot showing logP values of each fuel. logP values of each fuel were calculated using the software *molinspiration*.

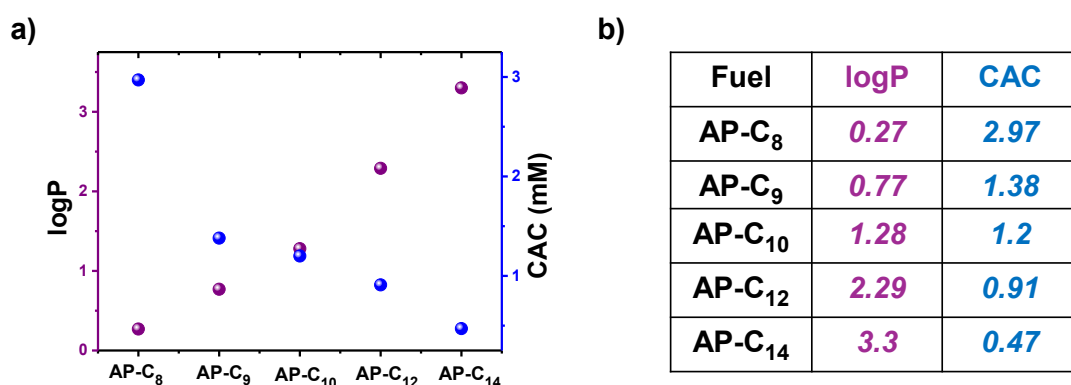


Figure S3.3: a) Plot showing the CAC and logP values of each fuel. b) Table showing the CAC and logP values.

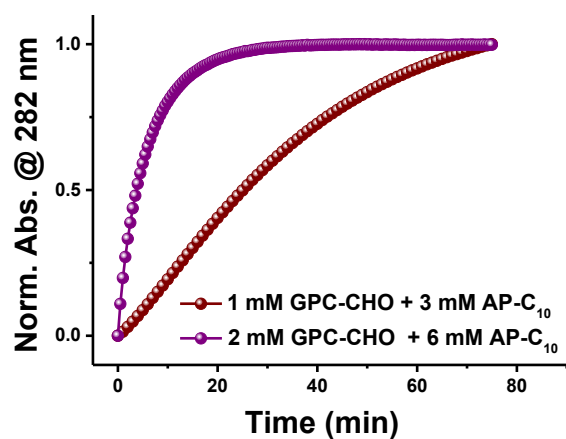


Figure S3.4: UV-Vis absorbance changes at $\lambda = 282$ nm showing the anabolic formation of GPC-Imine (1 mm cuvette) with varying AP-C₁₀ concentration.

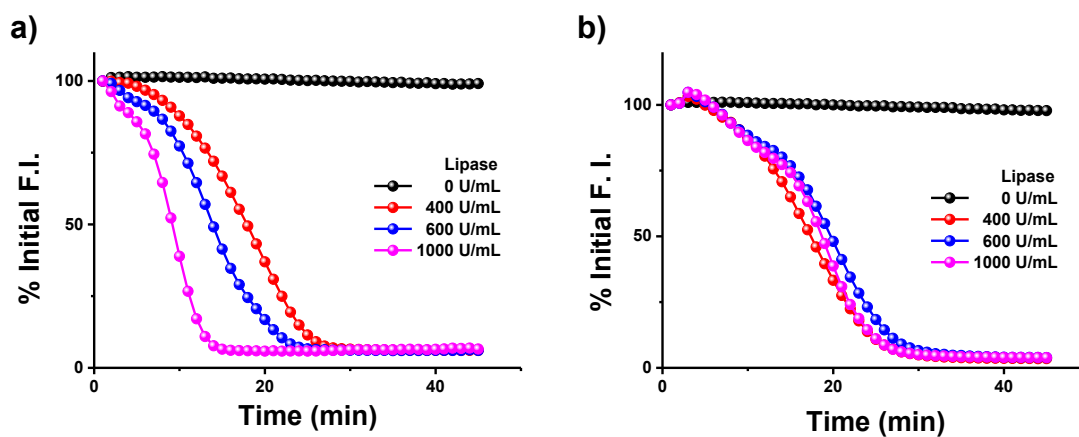


Figure S3.5: Localization of hydrophobic dye Nile red and its release profile from the a) GPC-Imine-AP-C₁₀ and b) GPC-Imine-AP-C₁₄ phospholipid vesicles.

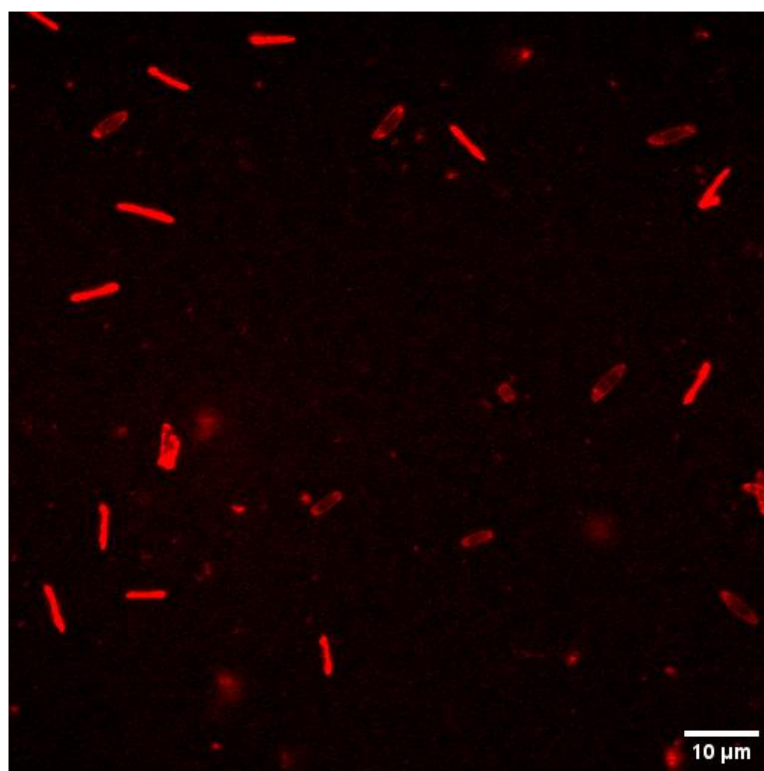


Figure S3.6: Additional confocal image showing the rugby ball like vesicles

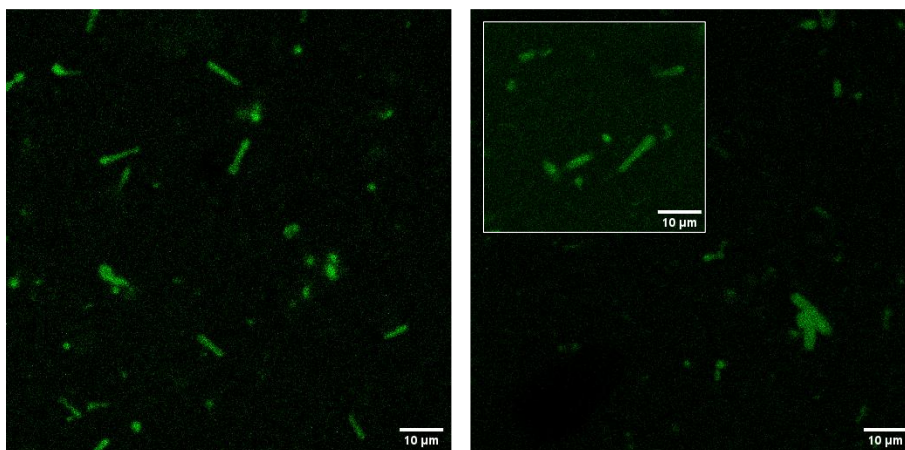


Figure S3.7: Confocal showing the aqueous interior of rugby ball like vesicles by encapsulating hydrophilic dye HPTS.

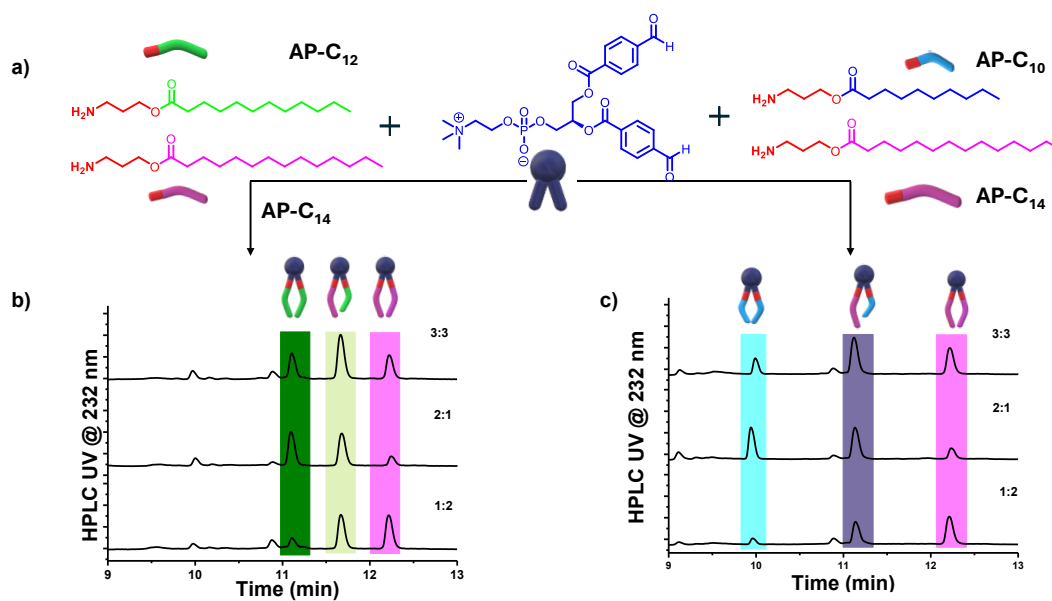


Figure S3.8: a) Schematics showing the addition of mix to different fuels to GPC-CHO. HPLC chromatograms of lipid compositions formed when GPC-CHO is mixed with b) AP-C₁₂ and AP-C₁₄ c) AP-C₁₀ and AP-C₁₄.

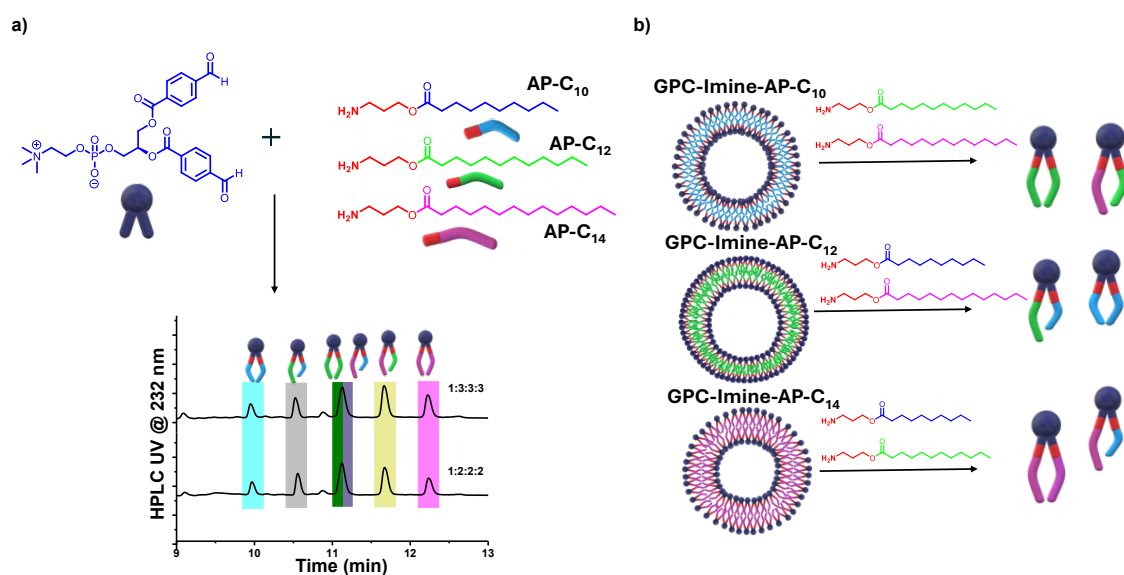


Figure S3.9: a) Schematics and HPLC chromatogram of lipids formed from the addition of premixed fuels AP-C₁₀, AP-C₁₂ and AP-C₁₄ altogether. b) Schematics showing the formation of distinct populations of lipids when multiple fuels are added to preformed GPC-imine vesicles. The mixed lipid populations C₁₀/C₁₄ and homo-lipid C₁₂/C₁₂ were indistinguishable due to their overlapping retention times.

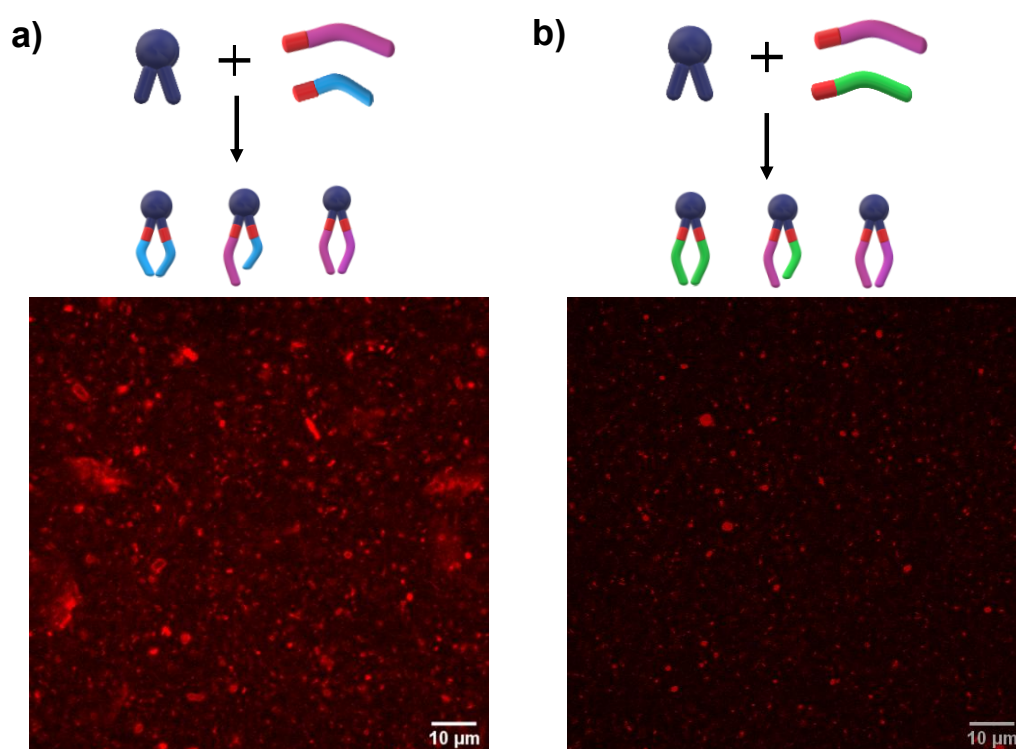


Figure S3.10: Confocal images showing the vesicular assemblies formed from lipid populations a) when AP-C₁₀ and AP-C₁₄ are added to GPC-CHO b) AP-C₁₀ and AP-C₁₂ are added to GPC-CHO.

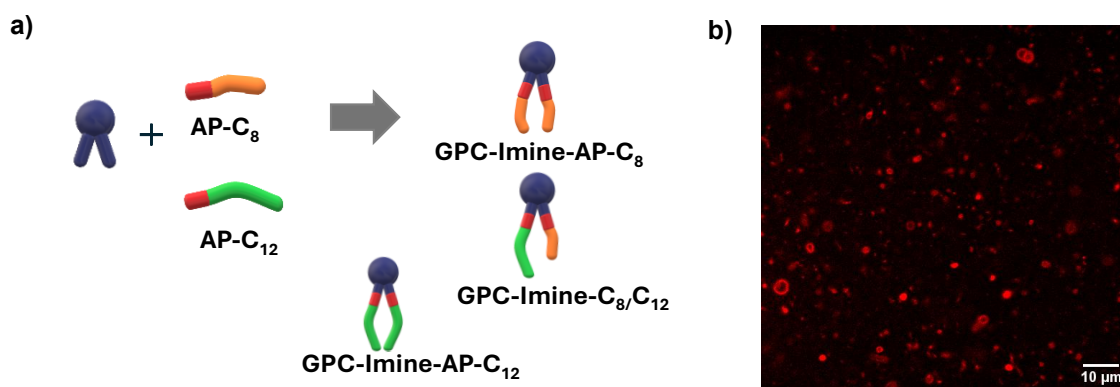


Figure S3.11: a) Schematic showing the formation of different lipid populations by mixing AP-C₈ and AP-C₁₂ fuels with GPC-CHO b) Confocal images showing the vesicles formed from the lipid populations GPC-Imine-AP-C₈, GPC-Imine-AP-C₈/C₁₂ and GPC-Imine-AP-C₁₂.

GPC-CHO	Molar ratio of Fuel			% GPC-Imine					
	AP-C ₁₀	AP-C ₁₂	AP-C ₁₄	ImineC10/10	ImineC10/12	ImineC10/14	ImineC12/12	ImineC12/14	ImineC14/14
1	3	0	0	100	0	0	0	0	0
1	2.5	0.5	0	60.15	34.63	0	5.20	0	0
1	2	1	0	36.50	47.25	0	16.24	0	0
1	1	2	0	10.78	34.74	0	54.48	0	0
1	0	3	0	0	0	0	100	0	0
1	1.5	1.5	0	22.59	47.49	0	29.91	0	0
1	2	2	0	13.44	44.75	0	41.80	0	0
1	3	3	0	14.77	45.65	0	39.56	0	0
1	1	0	2	8.60	0	38.98	0	0	52.41
1	2	0	1	38.99	0	46.36	0	0	14.64
1	3		3	15.95	0	48.67	0	0	35.36
1	0	1	2	0	0	0	9.99	45.22	44.77
1	0	2	1	0	0	0	33.21	33.15	33.63
1	0	3	3	0	0	0	25.63	25.46	48.90
1	2	2	2	8.58	13.77		34.31	25.40	17.90
1	3	3	3	11.69	14.92		30.93	26.44	15.99

Table S3.1: Percentage formation of individual lipid populations obtained when fuels at varying molar ratios were mixed with GPC-CHO. (*in HPLC, the lipid populations of C₁₀/C₁₄ and C₁₂/C₁₂ were indistinguishable due to their overlapping retention times*)

Imine Vesicles	Addition of fuel after 1h	% GPC-Imine					
		ImineC10/10	Imine10/12	Imine10/14	Imine12/12	Imine12/14	Imine14/14
GPC-C ₁₀	AP-C ₁₂	10.95	39.08	0	49.96	0	0
	AP-C ₁₄	17.75	0	41.56	0	0	37.97
GPC-C ₁₂	AP-C ₁₀	23.88	44.49	0	30.12	0	0
	AP-C ₁₄	0	0	0	27.19	22.97	47.21
GPC-C ₁₄	AP-C ₁₀	18.45	0	43.84	0	0	36.67
	AP-C ₁₂	0	0	0	22.39	28.27	47.05
GPC-C ₁₀	AP-C ₁₂ +AP-C ₁₄	6.85	17.74		37.21	25.84	12.34
GPC-C ₁₂	AP-C ₁₀ +AP-C ₁₄	5.20	15.08		34.86	30.24	14.60
GPC-C ₁₄	AP-C ₁₀ +AP-C ₁₂	8.38	15.43		36.74	30.03	9.39

Table S3.2: Percentage formation of individual lipid populations obtained when single or multiple fuels were mixed with pre-formed vesicles. Note that in HPLC analysis, the lipid populations of C₁₀/C₁₄ and C₁₂/12 could not be distinguished due to overlapping retention times.

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Chapter 4. Conclusions and Future Outlook

In recent years, supramolecular systems chemistry has gained momentum for its ability to emulate hallmarks of living matter, where continuous energy dissipation sustains adaptive and transient states.^[1-3] In this thesis, we extend these concepts to phospholipid vesicles, showing how fuel-driven formation, enzymatic degradation, and kinetic remodeling can recapitulate minimal features of membrane metabolism.

This thesis set out to explore how simple, water-soluble precursors can be transformed into phospholipids that assemble into vesicular structures under OOE conditions, inspired by the adaptive metabolism of biological membranes. Through systematic design of precursors, fuel-driven activation cycles, and enzymatic regulation, we have shown that phospholipid formation, degradation, and remodeling can be temporally programmed and tuned by molecular structure, hydrophobic interactions, and reaction kinetics.

Mimicking the autonomous functioning of biological membranes has been a central inspiration for this thesis. In living systems, energy-driven formation and degradation of phospholipid continuously remodels membranes, producing reversible structural changes that allow cells to adapt and evolve. Such dynamic turnover is at the core of dissipative adaptation and offers a powerful framework for understanding how simple molecular processes can give rise to complex life-like behaviour. This thesis builds on these ideas within the context of supramolecular systems chemistry, focusing on the design of OOE phospholipid assemblies from minimal, water-soluble precursors. By coupling fuel-driven lipid formation and enzymatic degradation, the work captures key features of natural membrane metabolism, growth, remodeling, and transient stability using simple chemical building blocks. In doing so, it bridges the gap between prebiotic systems chemistry and the construction of adaptive synthetic compartments that echo biological membranes.

A key outcome of this work is the formation of OOE phospholipid vesicles by coupling anabolic (imine formation) and catabolic (enzymatic degradation) processes, thereby mimicking the continuous turnover characteristic of living membranes. Importantly, chain-length mismatch introduced kinetic asymmetries that could bias vesicle composition under OOE conditions, providing the first experimental evidence that dissipative lipid remodeling can break the symmetry observed at equilibrium.

Beyond establishing chemical principles, this work points toward practical applications. The vesicles formed are uniform and of sub-100 nm size, compatible with drug delivery requirements. Encapsulation and release experiments with fluorescent probes confirmed functional compartmentalization and dynamic responsiveness. However, accumulation of waste products

limits long-term cycling. Addressing these will be crucial for advancing toward programmable, adaptive nanocarriers.

Our results establish a minimal yet powerful platform of dynamic liposomes, capable of fuel-driven remodeling and compositional adaptation. Unlike static carriers, these vesicles undergo continuous lipid turnover, making them dynamic similar to the living cell membranes. This dynamic reciprocity^[4] is likely to enhance communication between synthetic and biological membranes, offering new opportunities in biotechnology and nanomedicine. In particular, the ability of these vesicles to reorganize in response to external cues highlights their potential as next-generation delivery systems with improved cellular uptake and responsive release. Future efforts on the OOE lipids opens the way to on-demand nanomedicine and truly smart delivery platforms.

Moreover, the integration of such minimal phospholipid metabolisms into synthetic cell models provides a powerful route to mimic key aspects of cellular plasticity including adaptive remodeling, homeostasis, and selective response to external cues. Expanding the chemical scope of fuels and catalysts could yield more orthogonal control, while coupling lipid turnover with other functional modules (e.g., membrane proteins, signaling molecules) may bring us closer to autonomous protocell-like systems^[5-8].

This thesis demonstrates that phospholipid vesicles can be driven far from equilibrium, enabling dynamic remodeling and transient functions that echo biological membranes. Such systems differ fundamentally from classical equilibrium assemblies, where structural characterization at the end point is sufficient. Instead, OOE vesicles demand the ability to follow time-dependent transformations as assemblies grow, reorganize, and degrade under the influence of chemical fuels. As the field of supramolecular systems chemistry moves beyond static membranes toward adaptive, fuel-regulated vesicles, traditional techniques like dry-state TEM or diffraction-limited confocal imaging are no longer sufficient to fully capture their behaviour.^[9-10] Dynamic imaging is therefore crucial to understand how nonequilibrium chemical reaction networks reshape membrane architectures. Emerging tools such as fluorescence-based super-resolution microscopy, solution-state AFM, and liquid-phase TEM now provide real-time and in situ access to nanoscale dynamics. These approaches allow direct observation of vesicle remodeling, budding, and fission, while computational techniques such as molecular dynamics simulations complement experiments by offering atomistic insights into processes of self-assembly, that remain experimentally inaccessible. Looking ahead, the integration of these visualization techniques with OOE phospholipid systems will not only advance fundamental understanding of membrane dynamics but also accelerate the rational design of adaptive, life-like compartments for applications in nanomedicine and synthetic biology.

The next challenge is to move beyond minimal assemblies towards more autonomous, life-like systems. Future efforts will likely focus on integrating multiple reaction networks within same or different vesicles, enabling selective communication, feedback, and adaptation. By incorporating competing anabolic and catabolic pathways, such systems could sustain vesicle populations under OOE conditions, responsive to both internal and external cues. Also, bridging these synthetic assemblies with living systems, through targeted interactions with mammalian cells represents the next frontier, where vesicles evolve from model platforms into functional bioengineered compartments with therapeutic and diagnostic potential. The open-ended trajectory of this field suggests that such systems will not only deepen our understanding of supramolecular chemistry but also act as a bridge into synthetic biology and nanomedicine (Figure 4.1).

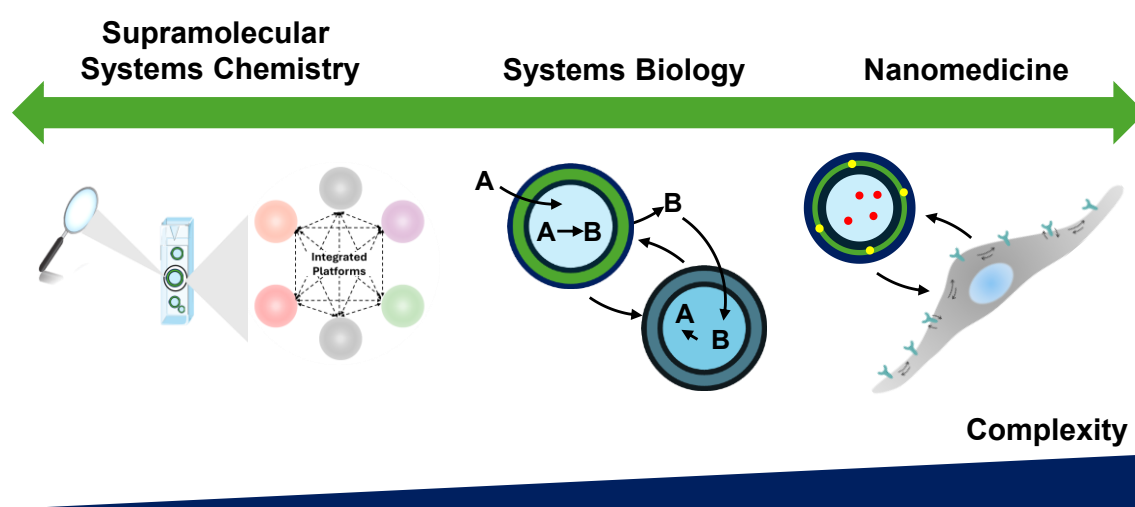


Figure 4.1. Open-ended trajectory highlighting how studies of OOE vesicles can enrich the field of supramolecular systems chemistry and evolve into synthetic biology and nanomedicine.

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1. **A. Venugopal**, L. Ruiz-Perez, K. Swamynathan, C. Kulkarni, A. Calò, M. Kumar. Caught in Action: Visualizing Dynamic Nanostructures Within Supramolecular Systems Chemistry, *Angew. Chem. Int. Ed.* **2022**, 62, e202208681.
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5. **A. Venugopal**, E. L. Nata, S. Ghosh, G. Battaglia, M. Kumar. Fuel Selection and Remodeling of Synthetic Out-of-Equilibrium Phospholipid Vesicles.
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