



**UNIVERSITÀ
DEGLI STUDI
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XXXVIII CICLO DEL DOTTORATO DI RICERCA IN

CHIMICA

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**Functional molecules and polymers for the creation of
life-like systems**

Settore scientifico-disciplinare: **CHIM/06**

**DOTTORANDO / A
FRANCESCO VICENTINI**

**COORDINATORE
PROF. ENZO ALESSIO**

**SUPERVISORE DI TESI
PROF. PIERANGELO GOBBO**

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ABSTRACT

Living systems rely on compartmentalization, non-equilibrium processes, and spatially organized chemical reactions to sustain complex functions. Reproducing these features in artificial systems represents a central challenge in protocell research. In this context, coacervates have emerged as promising models due to their high molecular density, dynamic responsiveness, and ability to sequester functional components and enhance chemical reactions. However, their intrinsic structural instability has so far limited their broader applicability.

This thesis investigates coacervate-based microcompartments as non-equilibrium protocellular systems capable of structural reconfiguration, selective molecular organization, and functional chemical activity. This work aims to establish coacervate microdroplets and coacervate vesicles as versatile platforms for the construction of active and adaptive protocells.

A mechanically driven, dissipative pathway is introduced to induce the reconfiguration of stable nano-coacervates into organized and robust coacervate vesicles, demonstrating a general and tunable strategy to overcome coacervate instability. This approach is extended to multiple polyelectrolyte systems, highlighting its broad applicability. In parallel, stable coacervate microdroplets are developed from functional synthetic polymers and employed as enzymatic microreactors, where chemical activity can be transduced into real-time fluorescent signals, enabling direct readout of non-linear enzymatic dynamics under non-equilibrium conditions. Beyond enzymatic systems, coacervate vesicles are functionalized with catalytic polyoxometalates to generate photocatalytic protocells capable of light-driven reactions, including water oxidation. The integration of catalytic activity, structural organization, and signal transduction within single compartments highlights the potential of coacervates as programmable microreactors.

Overall, this work demonstrates how non-equilibrium assembly, molecular integration, and functional design can drive the emergence of complexity in synthetic systems, positioning coacervate-based protocells as versatile platforms for protocell research.

RIASSUNTO

I sistemi viventi si basano sulla compartimentazione, su processi fuori dall'equilibrio e sull'organizzazione spaziale delle reazioni chimiche per portare a termine funzioni complesse. Replicare queste caratteristiche in sistemi totalmente artificiali rappresenta la sfida principale nella ricerca sulle protocellule. In questo contesto, i coacervati rappresentano modelli di protocellula promettenti grazie alla loro elevata densità molecolare, alla possibilità di rispondere dinamicamente agli stimoli esterni e alla capacità di sequestrare componenti funzionali e accelerare le reazioni chimiche. Tuttavia, la loro intrinseca instabilità strutturale ne ha finora limitato una più ampia applicabilità.

Questa tesi si propone di indagare sistemi protocellulari basati su coacervati fuori dall'equilibrio termodinamico. Questi sono in grado di riconfigurarsi strutturalmente, di sequestrare selettivamente molecole al loro interno, e di presentare attività chimica modulabile. L'obiettivo è stabilire coacervati e vescicole come strumenti artificiali versatili per la costruzione di protocellule attive e adattive.

Nella tesi viene introdotto un processo dissipativo basato sull'utilizzo di energia meccanica per indurre la riconfigurazione di nanocoacervati stabili in vescicole organizzate e robuste, dimostrando una strategia generale e modulabile per risolvere l'instabilità dei coacervati. Questo approccio è generalizzabile a diversi sistemi polielettrolitici, con conseguente ampia applicabilità. In parallelo, vengono sviluppati coacervati stabili a partire da polimeri sintetici funzionali, utilizzabili come microreattori enzimatici, in cui l'attività chimica può essere trasdotta in segnali fluorescenti in tempo reale. Oltre ai sistemi enzimatici, viene mostrato come le vescicole possano essere funzionalizzate con poliossometalati catalitici per generare

protocellule fotocatalitiche capaci di catalizzare reazioni guidate dalla luce come l'ossidazione dell'acqua. L'integrazione di attività catalitica, organizzazione strutturale e trasduzione del segnale all'interno di singoli compartimenti evidenzia il potenziale dei coacervati come microreattori programmabili.

Nel complesso, questo lavoro dimostra come l'assemblaggio fuori dall'equilibrio, l'integrazione di molecole complesse e la funzionalizzazione degli elementi che li costituiscono possano guidare l'emergere della complessità in sistemi sintetici, posizionando i coacervati come piattaforme versatili per la ricerca sulle protocellule.

ABBREVIATIONS

LLPS	Liquid-liquid phase separation
PEG	Polyethylene glycol
PDMAEMA	Poly(dimethylaminoethyl methacrylate)
PAA	poly(acrylic acid)
PDDA	Polydiallyldimethylammonium chloride
SANS	Small-angle neutron scattering
DSA	Dissipative self-assembly
DLS	Dynamic light scattering
Cryo-SEM	Cryo-scanning electron microscopy
FITC	Fluorescein Isothiocyanate
RITC	Rhodamine B isothiocyanate
FRAP	Fluorescence recovery after photobleaching
RAFT	Reversible addition-fragmentation chain transfer (polymerization)
PLys	Poly-L-lysine
GOx	Glucose oxidase
HRP	Horseradish peroxidase
o-PD	o-Phenylenediamine
2,3-DAP	2,3-Diaminophenazine
GPC	Gel permeation chromatography
PET	Photoinduced electron transfer

PTMAEMA	poly(2-(trimethylammoniummethyl methacrylate) chloride)
ICP-AES	Inductively coupled plasma atomic emission spectrometry
Ru₄POM	$[\text{Ru}_4(\mu\text{-O})_4(\mu\text{-OH})_2(\text{H}_2\text{O})_4(\gamma\text{-SiW}_{10}\text{O}_{36})_2]^{10-}$
PBI	Perylenebisimide
POM	Polyoxometalate
PTA	Phosphotungstic acid
PCV	Polyoxometalate coacervate vesicle
QS	Quantasome

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1 INTRODUCTION

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1.1 FROM SOFT MATTER TO LIVING SYSTEMS

Life represents the most intricate and sophisticated expression of self-organized matter. From molecular interactions to cellular processes, it embodies a continuous interplay between energy exchanges and structural organization. Understanding how such complexity arises from inanimate matter remains one of the fundamental scientific challenges, and reconstructing life-like features using synthetic components offers an unparalleled strategy to design new classes of adaptive materials.²

1.1.1 Life exists out of equilibrium

The second law of thermodynamics states that the entropy of an isolated system increases. In other words, such systems evolve spontaneously towards thermodynamic equilibrium, where entropy reaches its maximum (Figure 1.1a).

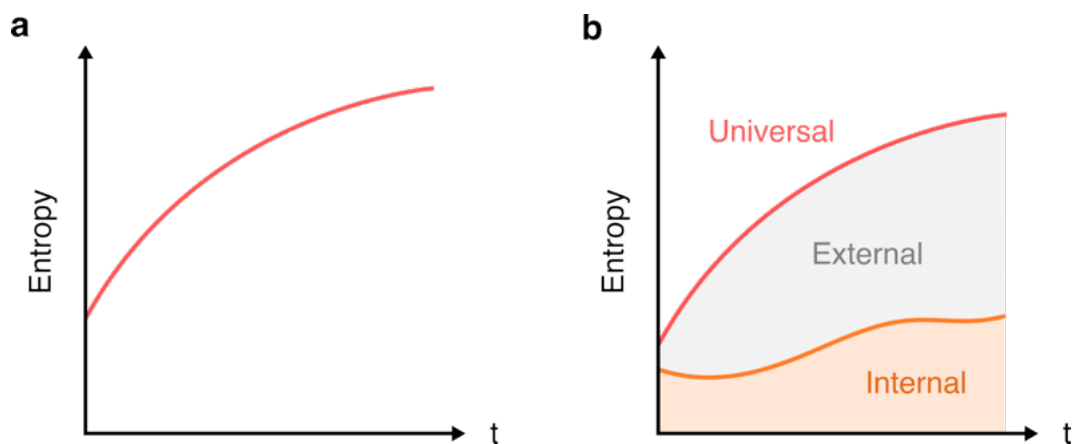


Figure 1.1 Schematic representation of entropy evolution in **a)** an isolated system approaching thermodynamic equilibrium, and **b)** an open, non-equilibrium system (dissipative) maintained by a gradual entropy exchange with the external environment.

Physical phenomena strictly follow this rule. Heat will always flow spontaneously from a hotter to a colder body until no temperature gradients remain. Similarly, gas

particles diffuse from regions of high pressure to regions of low pressure to balance pressure differences. However, this simple view of entropy does not capture the full scope of the second law, and its interpretation has been refined and extended over the years. In particular, when considering non-isolated systems, the picture becomes more complex.

“In a stationary state, the production of entropy inside a thermodynamic system with constant external parameters is minimal and constant. If the system is not in a stationary state, then it will change until the entropy production rate, or, in other words, the dissipative function of the system, takes the smallest value.”
– Prigogine, 1947.

In other words, non-equilibrium systems can maintain their form or structure by continuously dissipating energy into their surroundings.³ Dissipative systems can be viewed as gradient dissipators, which persist only by converting available free energy into entropy. This process occurs with a compensating increase in the entropy of the external environment, thereby preserving the overall increase dictated by the second law (Figure 1.1b). Both living and non-living systems rely on continuous fluxes of energy and matter from their environment to maintain organization and function in a locally reduced-entropy state.⁴

Living systems represent the clearest examples of open, non-equilibrium systems. An organism remains alive only by constantly exchanging energy and matter with its surroundings, sustaining a low-entropy state within itself. This principle lies at the core of Schrödinger’s reflections in *What is Life?*,⁵ where he famously introduced the notion of “order from disorder.” According to this view, life may be described as a dissipative structure that maintains its internal organization over time by exporting entropy to the environment.

Out-of-equilibrium thermodynamics manifests at every level of biological organization (Figure 1.2). Cells remain viable only as long as they sustain dissipative states; a transition to thermodynamic equilibrium would correspond to cellular death.

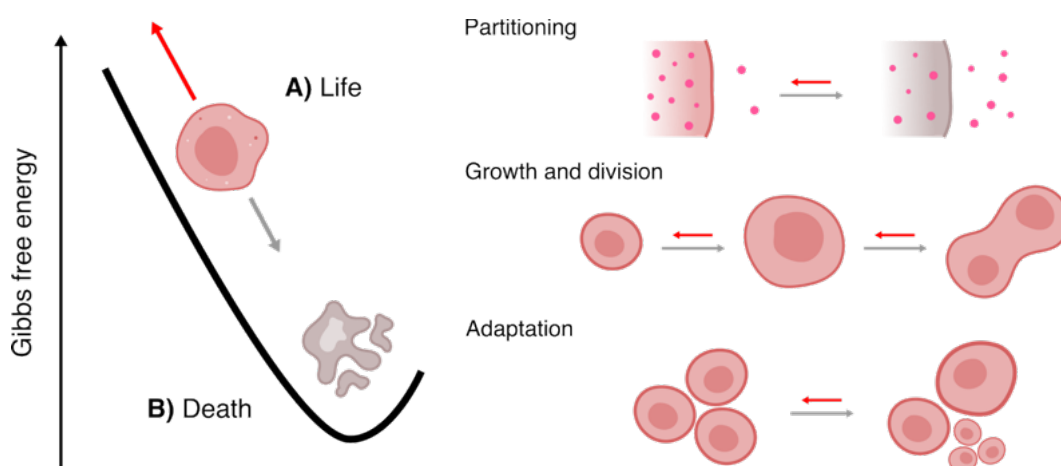


Figure 1.2 Schematic illustration of thermodynamic aspects of living systems. Living systems (A) exist only in an out-of-equilibrium dissipative state. An eventual thermodynamic equilibrium (B) would lead to cellular death. This aspect is reflected in many key biological functions represented on the right: partitioning of molecules and ions inside/outside the cells, growth and division of cells, and adaptation of living systems.

This principle governs a wide range of processes, from the establishment of ionic and molecular gradients across membranes, enabling selective transport and compartmentalization, to the growth, division, and adaptive responses of living systems. Understanding how such non-equilibrium behaviors emerge, and how they might be reproduced or harnessed in artificial systems, remains a central challenge for researchers striving to bridge the gap between soft matter and living matter.⁶

While thermodynamic equilibrium has been central to chemical theory since the origins of modern chemistry, non-equilibrium systems have only more recently emerged as a distinct research frontier. Their study has gained increasing attention over the past few decades owing to their inherent dynamism and their relevance across multiple length scales, from molecular reactions to supramolecular assemblies.⁷

1.1.2 Bio-mimetic materials

Natural systems have evolved over billions of years to optimize their responses to environmental constraints, material availability, and energy sources. The remarkable

diversity of structures and behaviors we observe today is the result of this continuous evolution. From a materials science perspective, living matter represents an extraordinary library of efficient, adaptive, and multifunctional designs that can inspire the creation of artificial systems.⁸ Scientists have sought to reproduce or reinterpret these natural models through bioinspired and biomimetic approaches.⁹ These strategies aim to transfer the organizing principles of living matter, such as self-assembly, adaptability, responsiveness, and hierarchical structuring, into synthetic materials and artificial systems.^{10,11} Rather than directly copying biological forms, these approaches often extract the physicochemical mechanisms that sustain dynamic behavior and integrate them into tunable artificial platforms.

In this context, research has exploited soft materials to emulate the dynamic organization characteristics of living systems. Polymers,^{12,13} colloids,¹⁴ and amphiphiles^{15,16} have provided versatile building blocks to construct systems that self-organize, adapt, or respond to environmental cues.^{17–20} Their intrinsic flexibility, reversible weak interactions, and sensitivity to external chemical and physical stimuli enable structural rearrangements and morphological transformations far from equilibrium.

More recently, these studies have evolved towards the design of active and dissipative soft materials, capable of converting external or chemical energy inputs into controlled motion, pattern formation, or transient self-assembly.²¹ By integrating feedback, fuel consumption, or reaction-driven reorganization, such systems begin to bridge the chemical landscape to the living one, offering simplified yet powerful models to explore the physical principles underlying life-like behavior.

1.2 BOTTOM-UP SYNTHETIC BIOLOGY

Cells are the fundamental building blocks of life.²² While biology and biotechnology investigate the structure, functions, and mechanisms of cells, the research has moved towards new directions, with the objective of taking inspiration from cells to create artificial alternatives.^{23,24} How can one take advantage of the dynamics and functions of natural cells, but build more robust and easily controlled artificial cells? In recent years, the field of bottom-up synthetic biology research has emerged to address this question by constructing simplified, artificial analogues of living cells from basic isolated “non-living” molecular components. Such systems serve as biomimetic platforms to study fundamental cellular properties, explore the dynamics of minimal life-like assemblies, and develop new technologies in place of biological cells.

Before constructing an artificial cell, it is essential to clarify what defines a cell. The chemoton model proposed by Tibor Gánti provides a theoretical framework for this purpose.²⁵ A minimal cell-like entity must combine three features: (1) a boundary system such as a membrane, (2) an internal metabolism or chemical motor, and (3) an information system represented by specific (bio)molecules (Figure 1.3). Achieving all three functions in one synthetic construct remains a major challenge. However, many research groups have created several options of artificial systems that can recreate the simple characteristics of living organisms.^{26,27} These systems have been termed artificial cells or protocells.

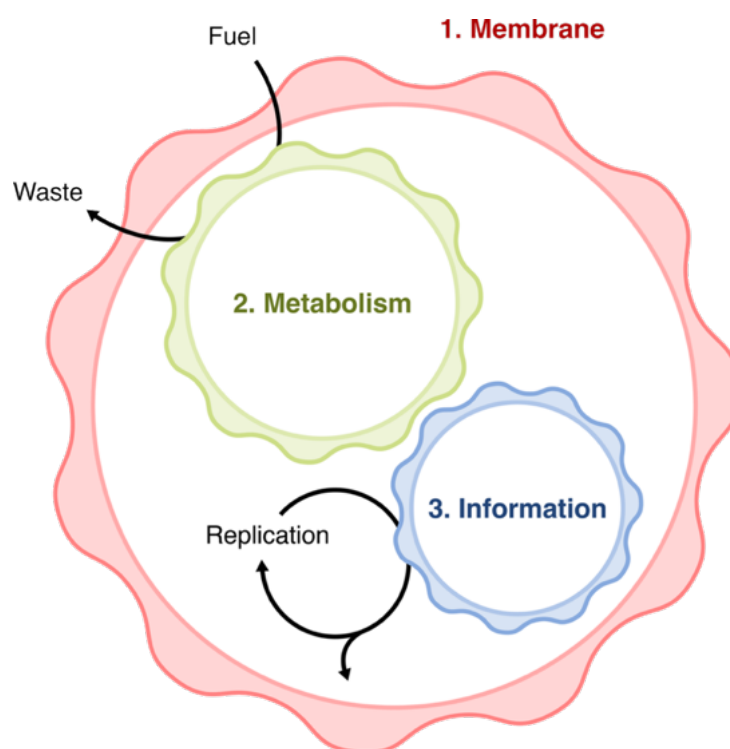


Figure 1.3 Schematic representation of the chemoton model proposed by Gánti.

Because the bottom-up approach builds an organization from molecular components, a wide variety of routes can lead to protocell formation. “Non-typical” protocells often mimic selected biological functions without being constrained by natural architectures.²⁸ As a result, membranes can be composed of polymers or proteins instead of the more natural phospholipids,^{29,30} feature non-classical metabolic networks with alternative reaction chemistries,^{31,32} or include engineered signal transduction pathways that convert external stimuli into controlled chemical responses.^{33,34} Likewise, the molecular systems responsible for information storage and communication can be reimagined through artificial analogues.^{35–37}

To navigate among the diverse families of protocells reported so far, it is useful to classify them according to their material composition and mechanism of compartmentalization (Figure 1.4). Among the most extensively studied are vesicular systems based on amphiphilic molecules, such as liposomes.^{38,39} These represent the closest structural analogues to biological cells, as their phospholipid bilayer mimics the

architecture of natural membranes. Given that lipid molecules could plausibly have been synthesized under prebiotic conditions,⁴⁰ liposomes have also become central models for origin-of-life studies.⁴¹

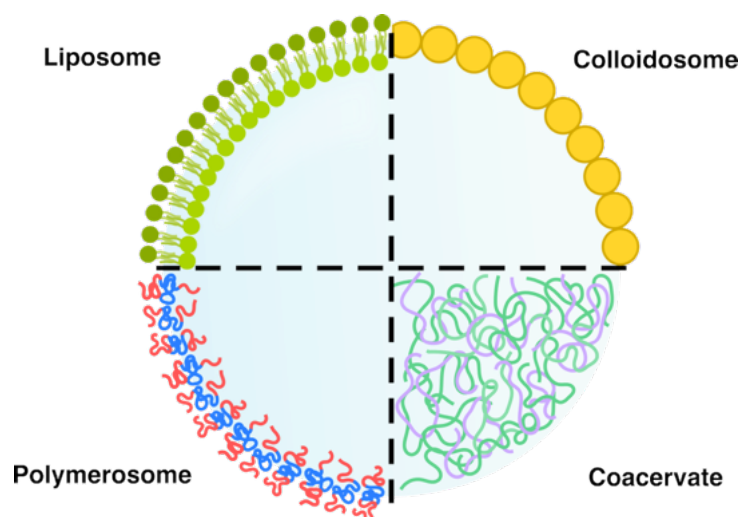


Figure 1.4 Schematic representation of different protocell models used in the field of bottom-up synthetic biology. Liposomes are membrane-bound using amphiphilic molecules, such as phospholipids; colloidosomes are made of self-assembled colloidal particles from w/o emulsions; polymersomes are formed *via* self-assembly of block copolymers in aqueous solution; and coacervates are liquid-liquid phase-separated droplets without a membrane.

Building on the same principles, other amphiphilic systems have been used as building blocks to generate protocells. Polymersomes, for instance, are usually assembled from block copolymers.^{42,43} These vesicles exhibit improved mechanical stability, tunable permeability, and enhanced chemical versatility thanks to modern polymerization and functionalization techniques, greatly broadening their potential for encapsulation and catalysis.⁴⁴

Similarly, colloidosomes can be assembled *via* spontaneous self-assembly of inorganic colloidal particles at water/oil interfaces.^{45,46} Their membranes, composed of tightly packed particles, present pores that allow selective permeability, creating dynamic boundaries with both structural and transport functionalities. In an analogous way, proteinosomes, formed by the assembly of protein-based nanoparticles in

emulsions, combine the modularity of colloidal systems with the biocompatibility of natural macromolecules.³⁰ These systems offer a unique level of programmability and can be engineered to display enzyme-like catalysis, communication, or adaptive responses.⁴⁷

Finally, in contrast to all these membranised structures, coacervates represent membrane-less protocells formed through liquid-liquid phase separation. Their dynamic internal organization, capacity for molecular concentration, and relevance to primitive biochemical processes make them particularly attractive as models for prebiotic and synthetic cellular systems. Given their importance to the present work, coacervates are discussed in detail in the following section (*vide infra*).

1.3 COACERVATES AS PROTOCELL MODELS

How cellular life emerged on prebiotic Earth remains one of the most profound and unresolved questions in science. In particular, understanding how a simple chemical world composed of small molecules evolved into complex, self-sustaining systems capable of Darwinian evolution continues to challenge researchers across disciplines. In the 1920s, Oparin proposed that life might have originated within tiny, membrane-less droplets formed through coacervation, the spontaneous phase separation of mixtures of charged or amphiphilic molecules.⁴⁸ These primitive compartments could have arisen from prebiotic molecules such as RNA, fatty acids, or small peptides suspended in the primordial environment.⁴⁹

Remarkably, similar phase-separated structures are still present within modern living cells. In eukaryotes, biomolecular condensates represent micrometer-sized, membrane-less compartments capable of selectively concentrating proteins and nucleic acids, thereby playing essential roles in metabolism, gene regulation, intracellular organization, and in some diseases.^{50–52}

Recent advances in the study of coacervation have shed light on its underlying physical and chemical driving forces, its relevance to both biology and prebiotic chemistry, and its potential as a model for constructing synthetic protocells. Yet, these discoveries have also opened new questions, inviting researchers to explore how such simple, dynamic assemblies could bridge the gap between non-living chemistry and the emergence of life.^{53,54}

1.3.1 Coacervates

Liquid-liquid phase separation (LLPS) is a thermodynamically driven physical phenomenon that occurs when macromolecules in solution condense into a liquid phase that coexists with the starting dilute phase, each with varying concentrations of components. LLPS can be found everywhere in nature. From the simple shaking of an

oil/water mixture to dress a salad to the complex regulation of enzymatic reactions inside living cells, where phase-separated condensates are crucial. Among the many examples, coacervates, dynamic membrane-free systems formed via spontaneous LLPS, have emerged as a frontier platform that mirrors both primordial protocell behaviors, cellular organelle functions and can be engineered to act as novel protocellular models.

Importantly, whether a solution undergoes phase separation depends strongly on the concentration and identities of the macromolecules in the solution and the environmental conditions, including temperature, salt type and concentration, co-solutes, pH, and the volume excluded by other macromolecules. There are two main types of LLPS: simple, when there is only one component in the liquid mixture, or complex, when two or more components are involved. Simple phase separation can usually form after changes in the solubility of the macromolecular species used, and usually it requires harsher conditions (*i.e.*, high temperature and high salt concentrations).^{55–57} For the purpose of this thesis, we will focus only on the complex mechanism. It can be categorized into segregative and associative (Figure 1.5 Complex liquid-liquid phase separation types and relative phase diagrams.). Segregative LLPS is a consequence of the immiscibility of the components involved in the process, as it happens for the PEG (polyethylene glycol)/dextran or protein/PEG systems.^{58,59} This process generates two aqueous phases, each enriched with one of the two components. On the other hand, associative LLPS (or complex coacervation) consists of the enrichment of two or more components in the same phase, forming complex coacervate microdroplets surrounded by a diluted water phase. Phase diagrams are generated by experiments that define the set of conditions that result in a single, well-mixed phase and the conditions that promote phase separation (Figure 1.5).

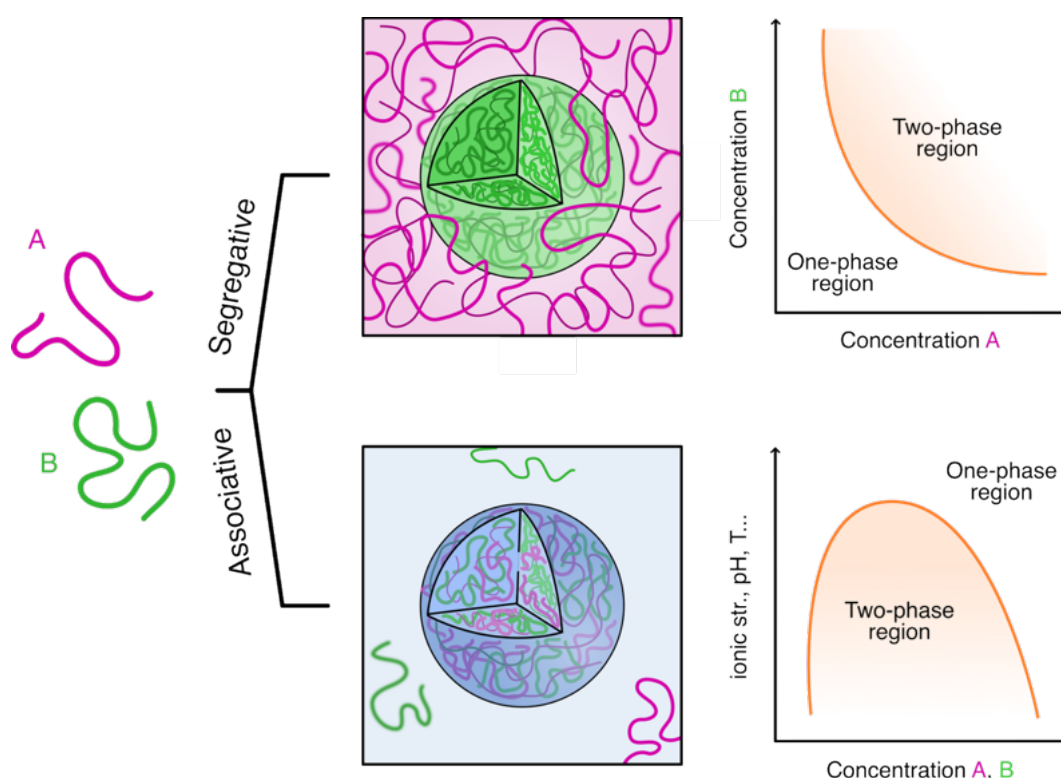


Figure 1.5 Complex liquid-liquid phase separation types and relative phase diagrams. In the phase diagrams, the orange area of the graph, named “two-phase region”, represents the conditions that favor LLPS.

Since that complex coacervation drastically depends on the properties of the species involved and their interactions, many different systems have been developed and studied since the first discoveries.⁶⁰ One feature that complex coacervate microdroplets have in common is their dynamicity, which is a direct consequence of the non-covalent interactions between the different components. In most cases, these interactions are nonspecific (e.g., electrostatic interactions, H-bonding, π - π stacking...), but coacervate droplets that rely on specific interactions have also emerged recently. The most general and ubiquitous example of complex coacervation regards the interaction of two oppositely charged polyelectrolytes, a polycation and a polyanion. This phenomenon was first theorized by Voorn-Overbeek.⁶¹ This model makes predictions for the phase behavior of polyelectrolyte complex coacervation, with phase separation being observed at low salt and polymer concentrations. Building

on these early findings, the research moved fast, and the theory has been expanded and improved to explain and analyze a vast number of different systems.⁶²

1.3.2 Artificial coacervate microdroplets

In living cells, biomolecular condensates provide a versatile strategy for compartmentalization, consisting mostly of disordered proteins and nucleic acids held together by weak electrostatic forces. Inspired by these natural assemblies, researchers have developed synthetic coacervates that reproduce key features of biomolecular condensates, including dynamic assembly and disassembly, internal fluidity, and selective, tunable sequestration of molecules.^{52,63,64} Common building blocks for artificial coacervates include polyelectrolytes, polypeptides, nucleic acids, and polysaccharides. In efforts to construct systems entirely *de novo*, synthetic polymers have become particularly attractive components for coacervate formation. Their chemical versatility allows precise control over properties such as charge density, hydrophobicity, and functional group display, making them ideal scaffolds for engineering phase behavior and compartmentalization.^{65–67} The physicochemical characteristics of these polymers, molecular weight, sequence patterning, and polarity, strongly influence their ability to undergo LLPS. To investigate how this aspect can govern coacervation, Spruijt *et al.* brilliantly studied the model system poly(dimethylaminoethyl methacrylate)/poly(acrylic acid) PDMAEMA/PAA by systematically varying the molecular weight and the salt concentration.⁶⁸ Their work established that electrostatic complexation occurs at the level of individual charged monomeric units, and that coacervate droplets can be viewed as dynamic clusters of reversible ionic bonds. The resulting complexes show viscoelastic properties distinct from those of their isolated components. Chain-length asymmetry introduces non-trivial effects: altering the polycation length modulates relaxation dynamics differently from altering the polyanion length. The temporary ionic cross-links underpinning these materials can be tuned through the addition of salt, which shifts the balance between bond formation and dissociation. To probe the internal structure and dynamics of the droplets, small-angle neutron and X-ray scattering experiments

revealed that polymer mobility and mesh size within the coacervate phase closely resemble those of the polymers in dilute solution, albeit with strong interpenetration of polycations and polyanions. Increasing salt concentration expands the mesh size, the average interpolymer spacing, consistent with previous observations in similar polymeric systems.⁶⁹

This intrinsic dynamicity is a defining characteristic of all LLPS-derived compartments. At the molecular ($\text{\AA}$) scale, proteins and nucleic acids within biomolecular condensates retain solution-like mobility, enabling rapid conformational fluctuations and fast reaction kinetics.⁷⁰ At the mesoscale, electrostatic interactions among charged repeating units drive droplet formation and stabilization. The ability to encode similar behaviors into synthetic polymers, from chain mobility to reversible cross-linking, is therefore central to the design of coacervate protocells. This capacity for chemical programming is precisely what makes polymer-based coacervate microdroplets so compelling as minimal models for studying the physical principles underlying biological compartmentalization.

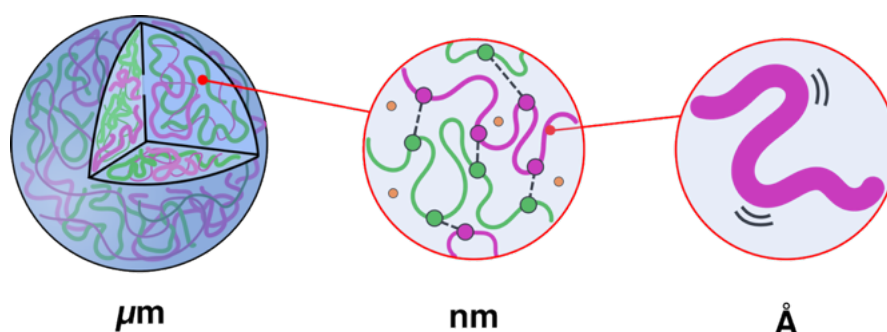


Figure 1.6 Scales and dynamics in biomolecular condensates and coacervates. At the mesoscale ($\mu\text{--nm}$), the dynamicity of the condensed phase is regulated by electrostatic interactions between polyelectrolytes and ions. At the molecular scale ($\text{\AA}$), polyelectrolytes retain their solution-like state with very fast motility and organization.

Artificial coacervate microdroplets can be functionalized to be controlled by stimuli such as pH, temperature, redox chemistry, and enzymatic activity.^{71–74} Dynamic coacervation is a highly desirable feature for cell-like microreactors and cell-like systems in general because it allows adaptive control of chemical processes through

selective sequestration of molecules by the coacervate droplets.⁷⁵ In addition, dynamic coacervation also allows control over structural properties such as size, morphology, and hierarchical organization, enabling the design of adaptive microreactors and cell-like systems.⁷⁶

While all these characteristics make them promising, coacervate microdroplets are intrinsically limited by their low interfacial tension and weak cohesion. These properties promote coalescence and surface wetting phenomena, which compromise structural stability and restrict their practical use as protocellular models or functional materials.^{77,78} To address these limitations and harness the beneficial features of coacervates, recent research has shifted toward developing membrane-bound coacervates.⁷⁹ Two principal approaches have emerged to achieve this goal. (a) The first relies on the introduction of external stabilizing agents that self-assemble at the coacervate-water interface, thereby providing mechanical reinforcement. (b) The second, more conceptually appealing strategy exploits intrinsic physicochemical interactions to induce a structural reconfiguration of the coacervate itself, leading to the formation of coacervate vesicles (Figure 1.7). While interfacial stabilization through external components is now relatively well established,^{80–84} the spontaneous or triggered formation of coacervate vesicles remains comparatively underexplored.

Coacervate vesicles constitute a distinctive hybrid architecture that integrates the molecular complexity, permeability, and responsiveness of coacervates with the compartmentalization and mechanical robustness typical of vesicular systems. These features make coacervate vesicles an exceptionally versatile and minimalist protocell model.

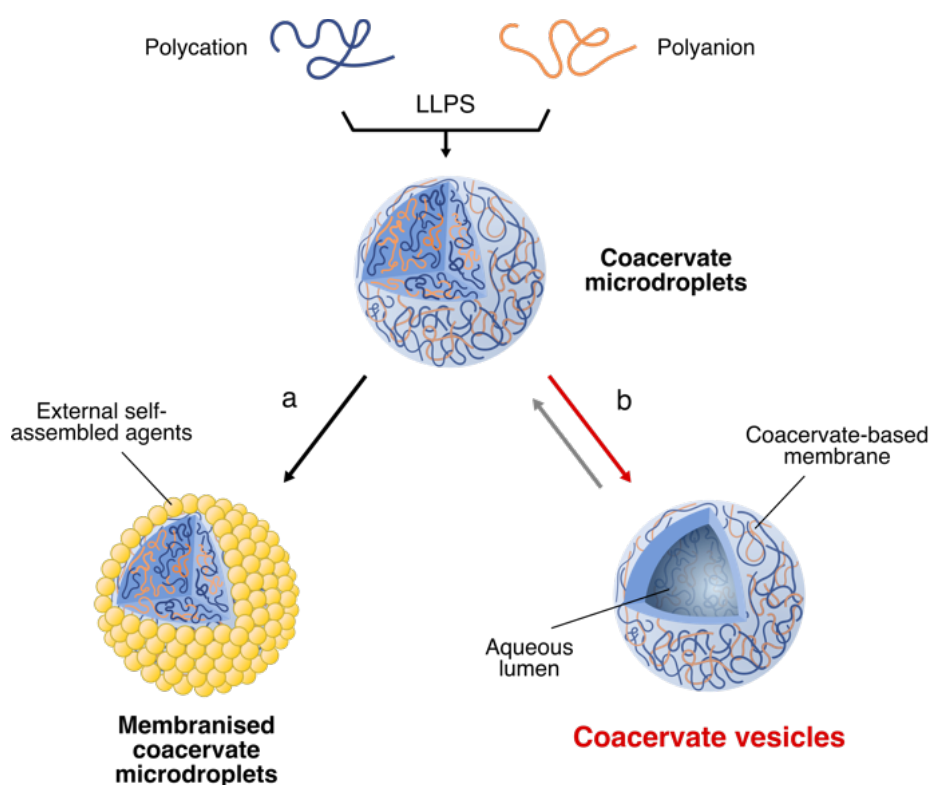


Figure 1.7 Schematic illustration of the two pathways to stabilize complex coacervate microdroplets. **a**, irreversible membranisation with external chemical agents (yellow spheres); **b**, reversible droplet-to-vesicle reconfiguration to form hollow coacervate vesicles.

1.3.3 From microdroplets to vesicles

Coacervate vesicles can be classified accordingly to their formation pathway from their droplet precursors into three categories: irreversible, reversible, and non-equilibrium (Figure 1.8). Irreversible coacervate vesicles arise from permanent structural reorganization, typically driven by strong electrostatic interactions or chemical modifications. Reversible vesicles, in contrast, remain dynamically interconvertible with their droplet precursors, enabling controlled switching between architectures. Non-equilibrium coacervate vesicles represent the most dynamic class, as their vesicular structure is maintained only under continuous energy input and collapses once the driving force is removed. These latter systems are particularly relevant as minimal models of active, life-like microcompartments.

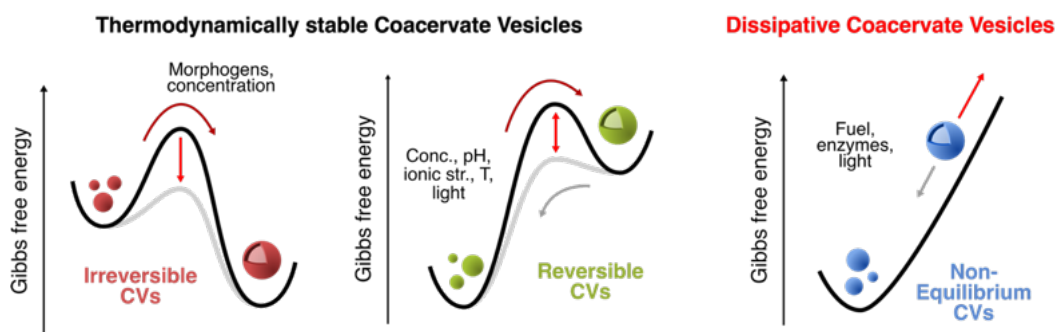


Figure 1.8 Schematic free energy diagrams illustrating irreversible, reversible, and dissipative self-assembly of coacervate vesicles. In each case, possible triggers for droplet-to-vesicle reconfiguration are reported.

1.3.3.1 Irreversible coacervate vesicles

Irreversible conversion strategies have been developed to overcome the inherent instability of coacervate droplets, yielding thermodynamically stable vesicles that do not revert to droplets under standard conditions. A widely adopted approach relies on the introduction of highly charged morphogenic agents that selectively bind one component of the coacervate, inducing spatial reorganization and vesicle formation.

A seminal example was reported by Williams *et al.*, who showed that phosphotungstic acid, a negatively charged polyoxometalate, strongly interacts with the polycation PDDA, transforming PDDA/ATP coacervate droplets into membrane-bound vesicles through osmotic pressure-driven reorganization.⁸⁵ The resulting structures feature a densely packed, birefringent membrane surrounding a coacervate-rich sub-shell.⁸⁶

This strategy has since been generalized through the use of mixed polyoxometalates to introduce catalytic functionality,⁸⁷ and through alternative morphogens such as surfactants or polyphosphates,^{88,89} which form strong salt bridges with positively charged components.

Irreversible vesicle formation can also be achieved without external additives by exploiting charge disproportion within the coacervate itself. An excess of one polyelectrolyte strengthens internal electrostatic interactions and generates osmotic gradients that spontaneously drive vesicle formation.^{90,91}

While these approaches yield robust structures, they do so at the expense of reversibility and dynamic adaptability.

1.3.3.2 Reversible coacervate vesicles

To retain the dynamic nature of coacervates, reversible vesicle systems have been developed that can switch between droplet and vesicle states in response to external stimuli. In these systems, the vesicular configuration represents a metastable state that can be accessed by modulating interaction strengths, while spontaneous reversion occurs once the stimulus is removed/reversed.

Stoichiometry plays a central role in this behavior.⁹² For example, RNA-rich RNA/peptide coacervates form stable, birefringent hollow vesicles that revert to droplets upon restoration of charge balance.⁹³ The underlying mechanism involves the formation of surfactant-like complexes under charge-imbalanced conditions, a behavior observed across multiple polyelectrolyte systems.⁹⁴

Reversible transitions can also be triggered by environmental stimuli such as pH and ionic strength,^{95,96} or temperature.^{97,98} These cues modulate electrostatic interactions within the coacervate and can even be generated internally. Notably, enzyme-containing coacervates have been shown to autonomously reorganize into vesicles through enzymatically induced pH changes, enabling signal-responsive behavior.^{71,99} Temperature-induced rearrangements in protein-based droplets similarly give rise to reversible vesicle formation.

Systems based on fatty acids and oppositely charged macromolecules can also undergo reversible vesiculation.¹⁰⁰ Concentration,¹⁰¹ pH,¹⁰² temperature,¹⁰³ reversible

chemical reactions,¹⁰⁴ and light¹⁰⁵ have been used to trigger vesicle formation. However, these assemblies often lose key coacervate characteristics such as internal fluidity and selective molecular uptake, behaving more like conventional lipid vesicles. As such, they fall outside the strict definition of coacervate vesicles despite their shared origin.

1.3.3.3 Non-equilibrium coacervate vesicles

Non-equilibrium coacervate vesicles differ fundamentally from equilibrium systems in that their vesicular architecture is sustained only through continuous energy consumption. These transient structures operate far from thermodynamic equilibrium and provide a conceptual bridge toward dissipative self-assembly processes observed in living cells.^{106,107}

Most reported examples rely on fuel-driven chemical reactions. In one representative system, carbodiimide fuel transiently modifies the polycation component of active coacervate droplets, generating osmotic imbalances that drive vesicle formation.¹⁰⁸ As the fuel is consumed and hydrolysis restores the original molecular state, the vesicles collapse back into droplets. Enzymatic systems follow similar principles. DNA nanostar coacervates, for instance, form vesicles when internal enzymatic cleavage produces smaller charged fragments that accumulate and generate internal pressure, a process sustained only while enzymatic activity persists.¹⁰⁹

Light can also serve as an energy input. Photoinduced polymerization within coacervate droplets increases internal charge density and osmotic pressure, leading to vesicle-like reorganization.¹¹⁰ Although such structures can persist for extended periods, they remain dissipative and ultimately revert to droplets once light stimulation ceases. Comparable behavior has been observed in flow-driven systems, where continuous polyelectrolyte influx sustains vesicle formation that reverses upon termination of the input.¹¹¹

1.3.4 Emerging applications of coacervate vesicles

The physicochemical characteristics of coacervate vesicles, directly related to their formation mechanism, underpin their functional behaviors. This property-function relationship not only clarifies current applications but also reveals a broad and still unfolding landscape of future opportunities (Figure 1.9).

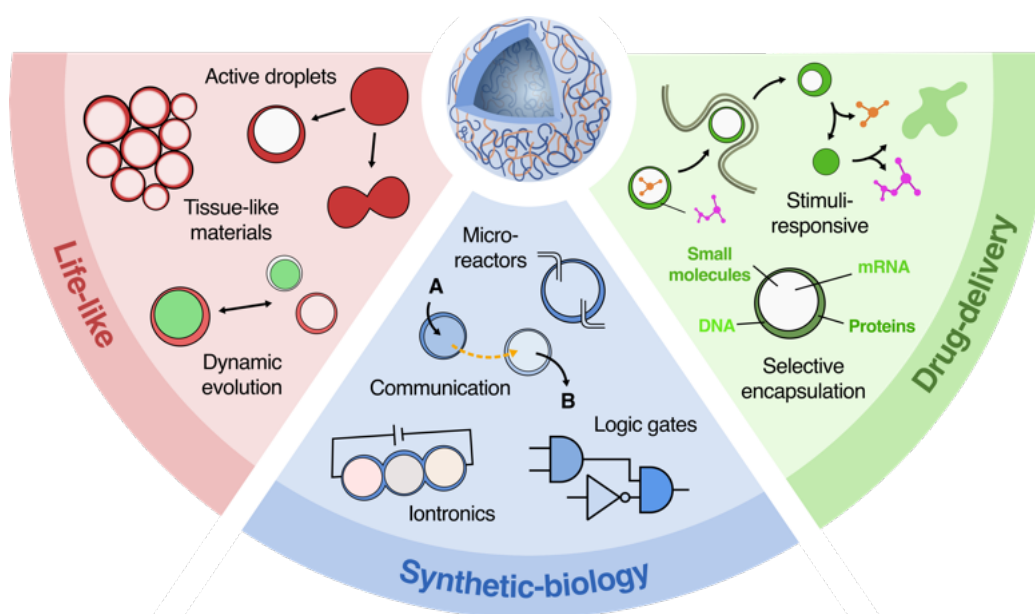


Figure 1.9 Schematic overview of current and potential future applications of coacervate vesicles in different areas: life-like models, synthetic biology applications, and drug-delivery compartments.

1.3.4.1 Life-like protocells

Since the early proposal by Oparin in the 1920s, coacervates have been considered plausible precursors to modern cells, providing a conceptual link between physicochemical processes and biological organization.^{48,112} However, the absence of a bounding membrane in classical coacervates leaves a critical gap in explaining the transition toward contemporary membrane-bound cells.

In this context, coacervate vesicles, particularly non-equilibrium systems, offer a more faithful representation of the dissipative nature of living matter. Active coacervate microdroplets have been shown to grow, divide,^{113,114} and it is therefore reasonable to envision the emergence of aqueous lumens and membrane-like shells from such dynamic droplets as a step toward primitive membranized protocells.^{108,115}

The compositional tunability of coacervate vesicles further enables selective partitioning of (bio)molecules between the coacervate shell and the internal aqueous compartment, allowing spatially organized chemical reactivity.⁹³ Reversible coacervate vesicles, in particular, support cycles of assembly and disassembly, providing a mechanism for the gradual acquisition, retention, and loss of functionality over time, a key prerequisite for evolutionary behavior.⁹⁸

Beyond individual compartments, the chemical identity of the coacervate vesicle membrane enables higher-order organization and communication. Irreversible coacervate vesicles have been shown to assemble into tissue-like structures through salt-bridge interactions involving membrane-associated polymers or polyoxometalates.^{89,116} Looking ahead, such interactions could be further extended through supramolecular recognition or covalent crosslinking strategies, opening routes toward protocellular assemblies with increasing structural and functional complexity.^{117,118}

1.3.4.2 Synthetic biology

Coacervate vesicles combine the inherent advantages of coacervates, such as selective molecular sequestration and reaction acceleration, with enhanced mechanical stability and modularity.^{119,120} As a result, they can be envisioned as micrometer-scale chemical reactors suitable for synthetic biology applications. Enzymes have already been selectively localized within the coacervate shell of polyoxometalate coacervate vesicles, which can be integrated into flow systems to support multistep catalysis and mimic simplified metabolic pathways.¹²¹ Beyond enzymatic systems, coacervate

vesicles assembled from inorganic catalysts have been shown to catalyze reactions in fully non-living settings, including catalase-like activity and photocatalytic water oxidation.¹¹⁶ Despite these advances, the integration of broader enzyme classes, artificial enzymes, or hybrid catalytic networks within coacervate vesicles remains largely unexplored and represents an important opportunity for future research.^{122,123}

The compartmentalization and responsiveness of coacervate vesicles also make them suitable platforms for protocell communication and collective behavior. Irreversible polyoxometalate-based vesicles have been incorporated into competitive protocell networks that exhibit collective responses to external stimuli.⁸⁷ Related concepts have been extended to gel-embedded coacervate systems that form tissue-like constructs capable of sensing and processing external signals.¹²⁴ Translating these behaviors into fully self-standing coacervate vesicles, without the need for external scaffolds, remains a key challenge. In this context, reversible and non-equilibrium coacervate vesicles offer additional programmability through their intrinsic sensitivity to stimuli such as light, pH, and ionic strength, suggesting potential applications as chemical logic elements in distributed computing systems.¹²⁵

1.3.4.3 Drug-delivery

Coacervate vesicles are also emerging as promising platforms for drug delivery, offering features that distinguish them from conventional lipid- or polymer-based carriers. Their biphasic architecture enables the simultaneous encapsulation of hydrophilic cargo in the internal aqueous lumen and charged or hydrophobic molecules within the coacervate membrane, facilitating multifunctional or combination therapies.¹²⁶ Their intrinsic responsiveness to environmental cues can be exploited for controlled or site-specific release.¹²⁷ For example, biocompatible nucleoprotein/DNA-based coacervate vesicles have been shown to selectively capture oncolytic viruses within their membranes and release them inside target cells.⁹⁰

Importantly, coacervate vesicles form spontaneously under mild, fully aqueous conditions, which is advantageous for the encapsulation of fragile biomolecules and contrasts with the complexity often associated with engineered delivery systems.¹²⁸ Notably, many bioactive molecules are themselves capable of undergoing LLPS, leading to the spontaneous formation of stable coacervate vesicles.⁹³ This property opens the possibility of self-contained carrier–cargo systems in which the therapeutic agent directly contributes to the structure and function of its own delivery vehicle.

AIM OF THE THESIS

Living systems rely on compartmentalization, non-equilibrium processes, and spatial organization of chemical reactions to sustain complex functions. Reproducing these features in artificial systems represents a central challenge in protocell research. In this context, complex coacervates offer a versatile platform due to their high molecular density, dynamic responsiveness, and ability to sequester functional components and enhance chemical reactions. Moreover, bottom-up design strategies enable precise control over coacervate composition, allowing chemists to tune their properties and potential applications as protocellular systems. Despite the growing interest in coacervates as models of minimal cells, their limited structural stability has constrained their broader applicability in protocell engineering and artificial life research.

The overarching aim of this thesis is to investigate coacervate-based microcompartments as non-equilibrium protocellular systems capable of structural reconfiguration, selective molecular organization, and functional chemical activity. By integrating concepts from soft matter chemistry, supramolecular self-assembly, and dissipative systems, this work seeks to establish coacervate microdroplets and coacervate vesicles as versatile platforms for the construction of active, adaptive, and functionally integrated protocells.

To achieve this aim, the following objectives were pursued:

- To elucidate a mechanically driven, dissipative pathway for the self-assembly of coacervate vesicles from non-equimolar nano-coacervates.
- To characterize the structural, dynamic, and reversible nature of these vesicles under non-equilibrium conditions.

- To design active coacervate microdroplets embedding enzymatic reaction networks and fluorescent transduction machinery, enabling real-time readout of non-linear chemical dynamics.
- To functionalize coacervate vesicles with catalytic polyoxometalates, enabling photocatalytic activity and protocellular communication.
- To develop fully catalytic and antenna-integrated photocatalytic protocells capable of light-driven water oxidation.

The main body of the thesis is structured as follows. **Chapter 2** focuses on mechanically driven dissipative coacervate vesicles and their use as protocells. **Chapter 3** presents the development of an active coacervate microdroplet system integrating enzymatic reaction networks with photoinduced electron transfer transduction machineries. **Chapter 4** explores polyoxometalate coacervate vesicles as photocatalytic protocells for light-induced water oxidation. Finally, the concluding chapter summarizes the main findings and outlines future perspectives.

Overall, this work aims to use the non-equilibrium nature of living systems as a guiding principle for the development of novel coacervate-based materials at the interface of soft matter chemistry and synthetic biology.

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2 MECHANICALLY DRIVEN DISSIPATIVE SELF-ASSEMBLY OF COACERVATE VESICLES

The work described in this chapter was published in F. Vicentini *et al.*, “Mechanical Energy Drives Dissipative Self-Assembly of Nanocoacervates into Vesicles with Cell-like Properties”, *Journal of the American Chemical Society* 2025, <https://doi.org/10.1021/jacs.5c14198>. This work was also presented at the 39th European Colloids and Interfacial Science – UK colloids 2025 conference, 7-12 September 2025, Bristol, UK, as an oral communication. The project was conducted under the supervision of Prof. P. Gobbo and Prof. W. H. Briscoe.

Cryo-SEM images were acquired in collaboration with M. Conti and S. Dal Zilio at IOM-CNR, Trieste, IT. We gratefully acknowledge Dr. Lauren Matthews (ISIS, Sans2d) and Dr. Leide Cavalcanti (ISIS, Zoom) for the technical support during the SANS experiment. SANS data obtained at ISIS are available at: <https://doi.org/10.5286/ISIS.E.RB2420467>.

2.1 INTRODUCTION

Self-assembly is a central mechanism through which Nature builds chemical complexity and organizes biological structure from relatively simple molecular building blocks.^{1–3} Two distinct thermodynamic pathways can give rise to self-assembled architectures.⁴ In the first pathway, assembly is driven by free energy minimization and leads to thermodynamically stable structures at equilibrium. Examples are the constructions of lipid bilayers, crystallization processes, and folding of proteins and nucleic acids.

The second route gives rise to out-of-equilibrium structures whose existence depends on the continuous consumption of energy. These transient assemblies gradually disappear once the energy input is removed.^{5–8} This more sophisticated form, known as dissipative (or dynamic) self-assembly (DSA), strictly mimics the adaptive, responsive, and regulated behavior of living systems.^{9,10} Biological examples include localized pH shifts or calcium ion concentration increases that can trigger the DSA of actin filaments or microtubules, which are essential for processes like cell division, movement, and intracellular transport.^{11,12}

Although equilibrium self-assembly has become a well-established and robust technique in modern science, the importance of mastering transient self-assembled structures through dissipative processes has only in recent years. DSA has been successfully used to generate a broad class of dynamic architectures, including nanoparticle aggregates,^{13,14} fibers,^{15,16} nanocrystals,^{17,18} vesicular nanoreactors,^{19,20} and active droplets.^{21–24} These developments point toward the possibility of engineering synthetic systems capable of adaptability, learning-like behaviors, controlled reactivity, and other higher-order functions regulated by external energy sources.^{25,26}

A variety of energy forms have been explored to sustain dissipative assembly, including chemical energy,²⁷ light,²⁸ electric fields,²⁹ and thermal energy.³⁰ Despite its simplicity, broad presence, and waste-free nature, mechanical energy has received comparatively limited attention, even though it is widely present in natural systems across the

mesoscale, from cellular processes to geological phenomena.³¹ Its use has primarily been restricted to assembling or disassembling structures at thermodynamic equilibrium,^{32–34} to oxidize chemical fuels and trigger DSA,^{35,36} or for the dissipative shear thickening of polymeric systems in solution.^{37,38} Reports where mechanical input directly generates well-defined dissipative structures remain notably scarce.

As outlined in Chapter 1, coacervate vesicles represent an emergent protocell model that merges the remarkable characteristics of complex coacervate microdroplets (*i.e.*, their ability to selectively concentrate and protect biomolecules,^{39,40} sustain diverse biochemical reactions,^{41,42} and dynamically evolve^{21,43}) with the enhanced stability and micro-compartmentalization provided by a vesicular architecture.⁴⁴ Previous approaches have generated such vesicles primarily through chemically triggered transformations of coacervate microdroplets.^{45–47} In contrast, the work described in this chapter focuses on a fundamentally different route: the use of impulsive mechanical energy to induce the DSA of coacervate vesicles from nano-coacervate precursors (Figure 2.1).

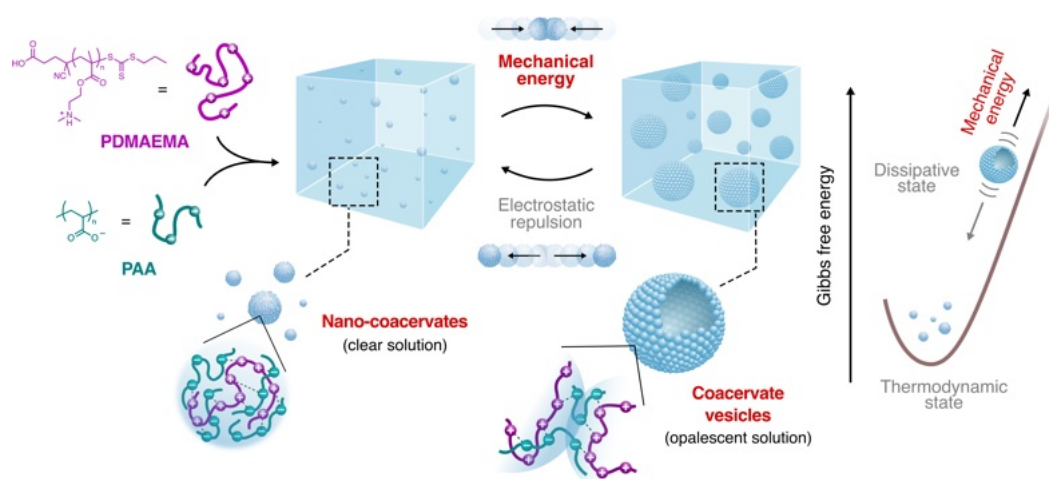


Figure 2.1 Scheme illustrating the mechanically induced DSA of coacervate vesicles. Upon mixing aqueous solutions of PDMAEMA and PAA (10 mM, pH 6.5) at a 1:4 molar ratio, the system forms a stable dispersion of negatively charged nano-coacervates that represent the equilibrium state. When an impulsive mechanical force is applied, these nanoscale droplets reorganize into micrometer-sized coacervate vesicles, a metastable dissipative state stabilized by interpenetration of the polyanionic chains and electrostatic contacts between adjacent nanodomains.

These mechanically generated vesicles can subsequently be chemically programmed to display hallmark cell-like features such as selective uptake of molecules, enzymatic reactivity, and communication based on diffusible chemical signals. Their dissipative character also makes it possible to control their structural evolution, for example, by promoting internal reorganization that enhances metabolic performance or leads to spatial segregation of catalytic species.

2.2 NANO-COACERVATES CAN SELF-ASSEMBLE TO FORM COACERVATE VESICLES

Coacervate vesicles were generated by applying an impulsive mechanical force to a dispersion of nano-coacervates prepared by mixing aqueous solutions of poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA, 20 kDa, monomers per chain $\approx$ 127, 10 mM, pH 6.5) and poly(acrylic acid) sodium salt (PAA, 2.1 kDa, monomers per chain $\approx$ 22, 10 mM, pH 6.5) (Figure 2.1). At near-equimolar PDMAEMA/PAA ratios, such mixtures typically yield micrometric, nearly neutrally charged coacervate droplets (phase diagram in Figure 2.1a, yellow region).⁴⁸ In contrast, at a 1:4 PDMAEMA/PAA molar ratio, we obtained a stable dispersion of negatively charged *nano*-coacervates with an average hydrodynamic diameter of 116 ± 40 nm (DLS) and a zeta potential of -44.0 ± 5.9 mV (Figure 2.1b, Figure S6). These nanodroplets remained thermodynamically stable over extended periods, displaying no measurable change in surface charge or signs of coalescence (Figure S6).

Cryo-scanning electron microscopy (Cryo-SEM) imaging confirmed the presence of discrete, non-coalescing spherical nanodroplets of around 100 nm in diameter at the 1:4 polymer ratio (Figure 2.2c), consistent with the DLS and zeta potential measurements. This behavior contrasts with the coalesced micrometer-sized droplets obtained at a 1:1 ratio (Figure S7). Small-angle neutron scattering (SANS) further highlighted the structural differences between these two regimes. At a 1:4 ratio, the scattering profile was well described by a core-shell oblate/spherical model, indicating

nano-coacervates comprising an internal complex coacervate core of radius ca. 35 nm surrounded by a highly solvated PAA-rich shell approximately 60 nm thick (Figure 2.2d, blue curve; Figure S8). This structure aligns with the negative surface charge and hydrodynamic sizes obtained independently. In contrast, the 1:1 ratio microdroplets (Figure 2.2d yellow plot, Figure S8) show strong clustering upon shaking, with overlapping polymer chains in conformations similar to those in semi-dilute solutions with a correlation length of 2.17 ± 0.04 nm, consistent with Spruijt *et al.*'s previous studies on similar systems.⁴⁹

Application of an impulsive mechanical force (1000–4000 Pa, corresponding to approximately 30 seconds of vigorous manual shaking) to the 1:4 nano-coacervate suspensions resulted in the formation of well-defined coacervate vesicles with a mean diameter of 11 ± 5 μm and a zeta potential of -51.3 ± 4.0 mV (Figure 2.2b,e,f). These structures exhibited the characteristic Maltese-cross birefringence under cross-polarized light (Figure 2.2e, Figure S10), consistent with the presence of an organized, densely packed membrane similar to those observed in liquid crystalline coacervates and polyoxometalate-stabilized vesicles.^{50–52}

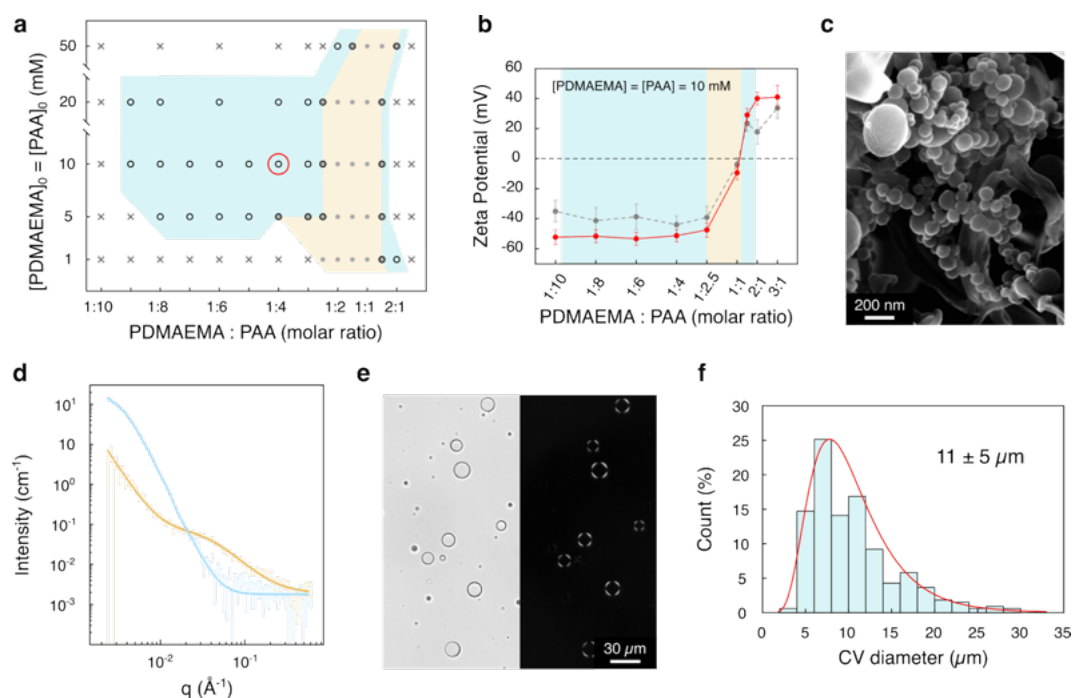


Figure 2.2 a, Phase diagram of PDMAEMA/PAA system ($\text{pH} = 6.5$) at varying volume ratios and polymer concentrations. The yellow region indicates coacervate microdroplets formation; in contrast, the azure region is referred to coacervate vesicle formation upon 30s of impulsive force application. The red circle marks the primary concentration and molar ratio used for the study. **b**, Zeta potential measurements comparing nano-coacervates (grey plot) and coacervate vesicles (red plot) assembled from 10 mM PDMAEMA/PAA solutions at different molar ratios. Error bars show standard deviation ($n = 3$). **c**, Cryo-SEM image of nano-coacervates formed at a 1:4 molar ratio. **d**, SANS analysis comparing 1:1 (yellow plot, coacervate microdroplets) and 1:4 (azure plot, coacervate nanodroplets) PDMAEMA/PAA (10 mM) molar ratio solutions after 30 s of shaking. **e**, Brightfield (left) and cross-polarized (right) microscopy images of coacervate vesicles. **f**, Size distribution analysis of coacervate vesicles showing an $11 \pm 5 \mu\text{m}$ average diameter.

The mechanically formed vesicles were metastable and gradually disassembled over approximately seven days yet could be reassembled repeatedly from the same solution by reapplying the mechanical force (Figure 2.3). Vesicle disassembly proceeded in distinct stages: a slow decrease in vesicle diameter over the first 24 hours, collapse of the internal aqueous lumen after roughly three days, and complete reversion to the initial nano-coacervate dispersion after about seven days (Figure S11).

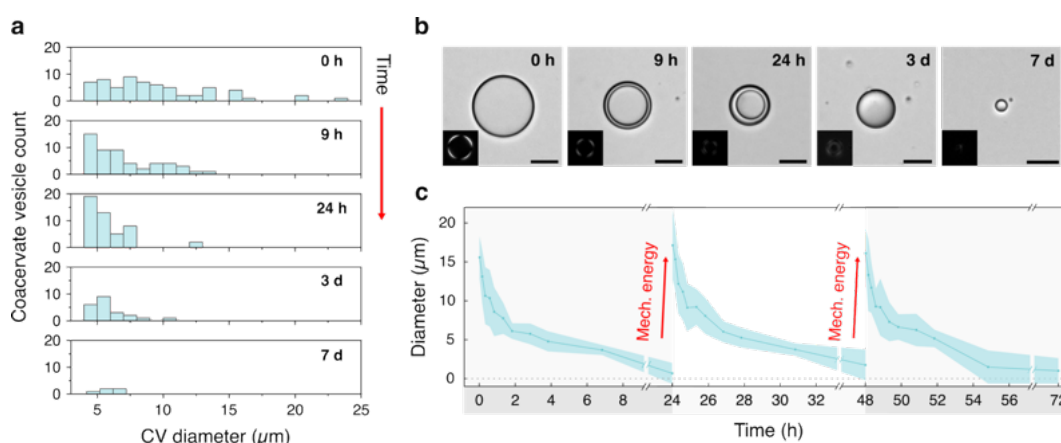


Figure 2.3 **a**, Time-dependent size distribution showing vesicle disassembly profile. **b**, Brightfield microscopy images showing vesicle disassembly. The cross-polarized microscopy insets show how the characteristic birefringence of coacervate vesicles is lost during membrane collapsing. Scale bars = 10 μm. **c**, Time-dependent changes in the average vesicle diameter. After dissolution (diameter = 0 μm), vesicles can be reformed upon application of mechanical energy. Error bands show standard deviation (n = 25).

Cryo-SEM imaging provided direct evidence for the membranized architecture of the mechanically assembled vesicles. The images revealed hollow structures surrounded by a membrane approximately 100 nm thick, composed of partially fused nano-coacervates arranged in a monolayer, with a characteristically rough surface texture (Figure 2.3a, Figure S7). Interestingly, the small dark parts on the membrane present a diameter around 50-100 nm, comparable with the size of the nano-coacervates' core. This morphology suggests a formation mechanism in which polymer chains interpenetrate sufficiently to mediate partial fusion and structural stabilization of individual nano-coacervates, yet without fully erasing their nanoscale identity. This interpretation is consistent with SANS measurements, which showed that the polymer-level structural organization at 1:4 PDMAEMA/PAA (10 mM) remains largely preserved after mechanical perturbation (Figure 2.2d, solid blue curve; Figure S8).

To examine membrane composition and dynamics, fluorescently labelled polymers (FITC-PDMAEMA and RITC-PAA) were incorporated into the forming vesicles and analyzed by confocal laser scanning microscopy. As shown in Figure 2.3b,c, the vesicles were hollow and exhibited strong colocalization of both polymers in the

membrane (Pearson's coefficient 0.851; Figure S12), indicating a homogeneous mixed-polymer membrane at the microscale. In parallel, fluorescence recovery after photobleaching (FRAP) experiments (Figure 2.4d,e) revealed a marked reduction in polymer mobility within the vesicle membranes.

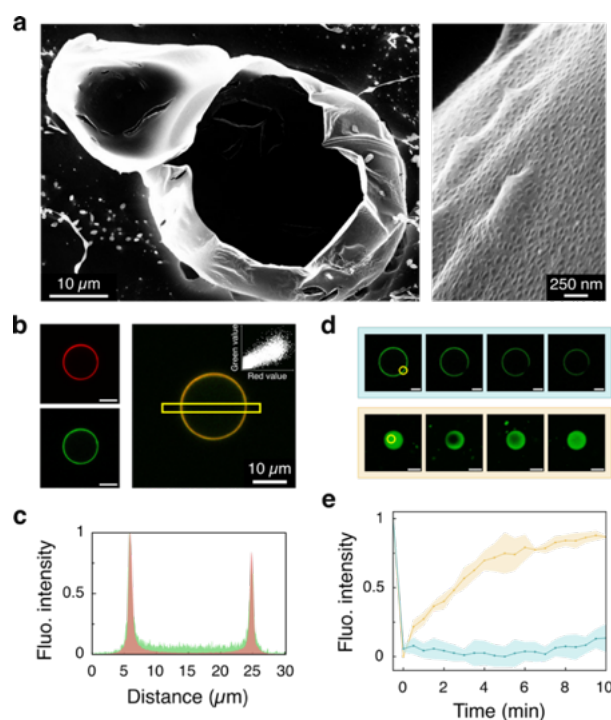


Figure 2.4 Structural characterization of coacervate vesicles. Cryo-SEM image of a sectioned coacervate vesicle showing its hollow interior; inset: magnified membrane composed of partially fused nano-coacervates. **b**, Confocal laser scanning microscopy images of a single coacervate vesicle assembled at a 1:4 ratio of PDMAEMA/PAA (10 mM), the polymer solutions contained 10 mol% FITC-tagged PDMAEMA (green) and 10 mol% RITC-tagged PAA (red), respectively. The merged image (right) shows polymer colocalization, confirmed by pixel data scatter plot inset (Fig. S14). Scale bars = 10 μm . **c**, Normalized fluorescence intensity profiles (green/red channels) along the yellow square in **b**. **d**, FRAP analysis of vesicles (top) and standard coacervate microdroplets (bottom), shown at pre-bleach, $t = 0$ s, 2 min, and 10 min. The yellow region indicates the bleached area. Scale bars: 5 μm . **e**, Normalized fluorescence recovery curves corresponding to **d** for microdroplets (yellow) and vesicles (blue). Vesicles display no recovery over 10 min, indicating low polymer mobility. Error bands represent $\pm$ s.d. ($n = 3$).

2.3 IN-DEPTH CHARACTERIZATION AND GENERALITY

PDMAEMA-PAA nano-coacervates are held together by strong electrostatic interactions between the two charged species. The formation of networks of these nanoscale objects to form organized coacervate vesicles is a direct consequence of how this electrostatic interaction prevails against the external mechanical energy applied. It has been previously reported how the formation of complex structures of oppositely-charged polyions in solution depends on various factors, besides charge stoichiometry, concentrations, degrees of polymerization, length ratio of the charged segments, and charge densities. Other effects of the surrounding medium, most importantly the pH and the ionic strength, can influence the structure.⁵³ Schatz *et al.* firstly reported how mixtures of chitosan and dextran sulfate could lead to form core-shell structures.⁵⁴ Sæther *et al.* further extended this observation, reporting how oppositely charged alginate and chitosan, chosen as simple polyionic models, could form core-shell nanometric polyelectrolyte complexes with varying size and charge.⁵⁵ The smallest and most organized particles were obtained by adding one of the two polymer solutions into an excess amount of the other solution and by using forms of the component polymers with lower molecular weights.

Similarly, we investigated how variations in the molecular weight of the two polyionic components influence both the properties of the initial nano-coacervates and the final formation of coacervate vesicles. To this end, a series of PDMAEMA polymers with different molecular weights (10, 20, 40, and 80 kDa) were synthesized via RAFT polymerization and combined with poly(acrylic acid) samples of varying molecular weight (2.1, 5.1, and 8 kDa), while maintaining a constant 1:4 PDMAEMA/PAA molar ratio. DLS measurements revealed a systematic decrease in particle size with increasing PAA molecular weight, whereas an increase in particle size was observed upon increasing the molecular weight of PDMAEMA (Figure 2.5).

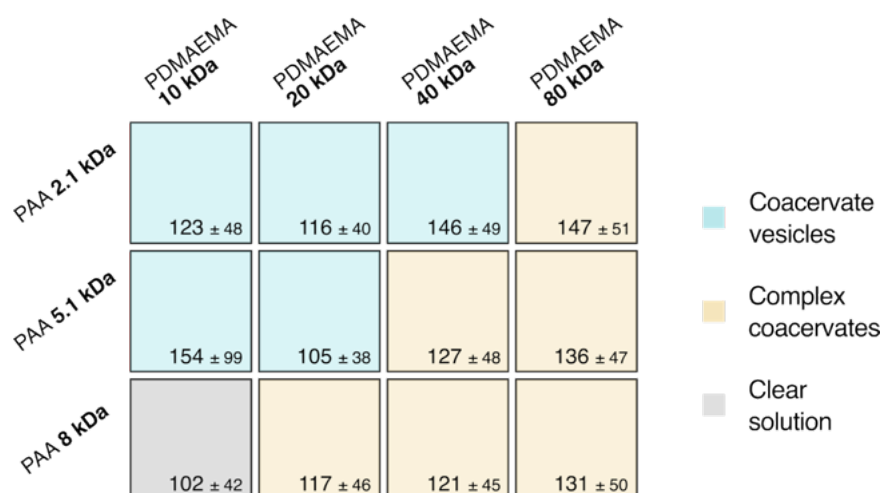


Figure 2.5 Table showing how variations in the molecular weight of the polyions affect the final outcome of the system. For each combination at a 1:4 PDMAEMA/PAA molar ratio, the macroscopic appearance of the system after 30 s of manual shaking and the hydrodynamic diameter determined by DLS (nm) are reported. As expected, the particle diameter decreases with increasing PAA molecular weight. Vesicle formation was observed only for combinations involving lower-molecular-weight PDMAEMA and PAA.

Preliminary SANS experiments, which are currently ongoing, support these observations. In particular, assemblies formed from higher-molecular-weight polyions were more difficult to describe using a simple core-shell model, suggesting the emergence of alternative or more complex internal architectures. Importantly, these trends at the nanoscale are reflected in the macroscopic outcome of the system upon application of mechanical force. Coacervate vesicles were preferentially formed when both polyionic components possessed relatively low molecular weights, whereas higher-molecular-weight combinations predominantly yielded coacervate microdroplets or disordered aggregates (Figure 2.5). These results underscore the critical role of polymer molecular weight in governing not only the size and structure of nano-coacervates but also their ability to reorganize into higher-order vesicular architectures.

The formation of coacervate vesicles through impulsive mechanical input was not restricted to the 1:4 PDMAEMA/PAA composition but extended across a broader

range of polyanion-polycation ratios. The azure region in the phase diagram (Figure 2.2a) highlights the compositions that yielded vesicles after force application (Fig. S15).

All these observations support a mechanism in which mechanical energy temporarily overcomes the stabilizing forces acting on the nanodroplets. The applied impulsive force (1000-4000 Pa) exceeds the electrostatic and osmotic barriers (1500-2200 Pa) that normally prevent coalescence, enabling close contact between droplets and promoting chain interpenetration and entanglement (Figure 2.1). Importantly, an impulsive force was required; shear-based agitation such as stirring, vortexing, or sonication did not generate vesicles (Figure S9). Vesicle formation only occurred when an excess of polyanionic or polycationic chains was present at the droplet surface, which explains why the effect was confined to compositions outside the classical coacervation window (azure region in Figure 2.2a). However, the long-term fate of the systems is determined by thermodynamics. Chain interpenetration during vesicle formation increases local monomer concentration and charge density within the membrane, generating a heightened osmotic pressure. Over time, the system relaxes toward a lower Gibbs free energy state: polymer chains gradually disengage, and the vesicles revert to the original, stable nano-coacervate dispersion.

To further assess the generality of this mechanically driven DSA, two additional polyelectrolyte systems were examined: poly(diallyldimethylammonium chloride)/polyacrylic acid (PDDA/PAA; Figure 2.6a) and poly-L-lysine/double-stranded DNA (PLys/dsDNA; Figure 2.6b). Both systems are known to form coacervate droplets at defined mixing ratios (yellow regions). Upon applying impulsive mechanical force outside these ranges, we again observed vesicle formation (azure regions), accompanied by birefringent optical textures characteristic of organized membranes. This confirms that the mechanism is not specific to PDMAEMA/PAA. In the PDDA/PAA system, however, vesicles did not form under conditions of excess PDDA, likely due to the large molecular weight difference between PDDA (≈ 100 kDa) and PAA (2.1 kDa), which reduces the ability of the bulkier polycation chains to interpenetrate during droplet fusion.

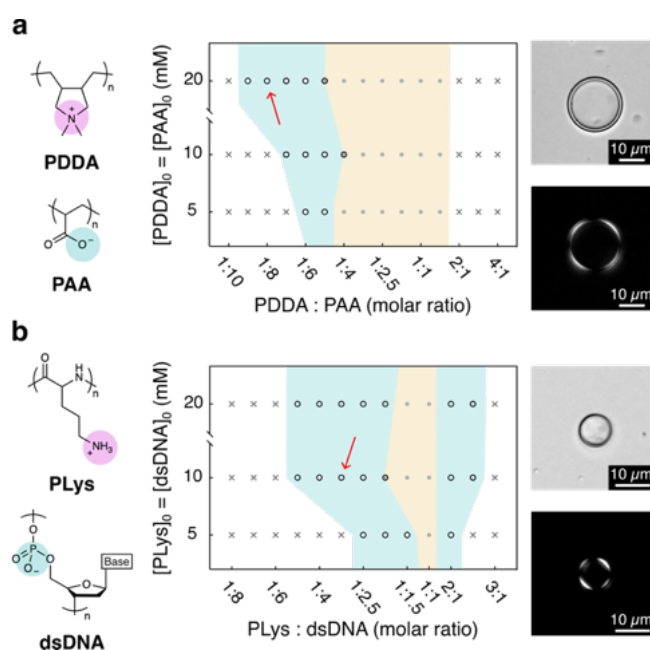


Figure 2.6 a, Molecular structures and phase diagram for the PDDA/PAA system. The red arrow indicates the conditions (1:8 molar ratio, 20 mM) used to acquire the brightfield (top) and cross-polarized (bottom) microscopy images on the right showing a typical birefringent single vesicle. **b**, Molecular structures and phase diagram for the PLys/dsDNA system. The red arrow indicates the conditions (1:3 molar ratio, 10 mM) used to acquire the brightfield (top) and cross-polarized (bottom) microscopy images on the right showing a typical birefringent single vesicle. The pH of all solutions was adjusted to 6.5.

2.4 COACERVATE VESICLES AS PROTOCELL MODELS

We first examined whether coacervate vesicles could selectively sequester guest molecules from their surroundings.⁵⁶ Vesicles were assembled in the presence of different fluorescent dyes, and their distribution was quantified by confocal laser scanning microscopy (Figure 2.7a, Table S4). Dye localization depended primarily on molecular hydrophobicity and charge at pH 6.5. Hydrophobic dyes with high logD values (e.g. Nile Blue) preferentially partitioned into the membrane, whereas weakly hydrophobic, positively charged species (e.g. $[\text{Ru}(\text{bpy})_3]^{2+}$) accumulated in the internal

lumen. Strongly anionic and hydrophilic molecules (e.g. Calcein) showed little or no uptake. This selective sequestration highlights the potential of coacervate vesicles to integrate photocatalytic dyes, enzyme cofactors, or drug-like molecules in a spatially organized fashion. These observations are in line with the model we attributed to the nano-coacervates, with a more hydrophobic core surrounded by an anionic shell. During the mechanically induced vesicle formation, the components that, on paper, can interact strongly with the nano-coacervates are the ones remaining inside the membrane after the DSA.

We next combined this uptake behavior with the mechanically driven dissipative assembly mechanism to generate protocells with rudimentary metabolism and communication abilities. Nano-coacervates containing either rhodamine B isothiocyanate (RITC)-labelled glucose oxidase (GOx) or fluorescein isothiocyanate (FITC)-labelled horseradish peroxidase (HRP) were first prepared (step I, Figure 2.7b). After applying impulsive force, these enzyme-loaded nanodroplets assembled into two distinct protocell populations. Confocal imaging showed clear spatial segregation: HRP accumulated in the lumen, while GOx localized in the membrane (Figure 2.7c,d). This distribution follows the physicochemical properties of the proteins. GOx (160 kDa), more hydrophobic and negatively charged at pH 6.5, behaved similarly to Eosin Y and partitioned into the membrane. HRP (44 kDa), smaller, positively charged, and less hydrophobic, accumulated in the lumen, consistent with the behavior of $[\text{Ru}(\text{bpy})_3]^{2+}$ (Table S5).

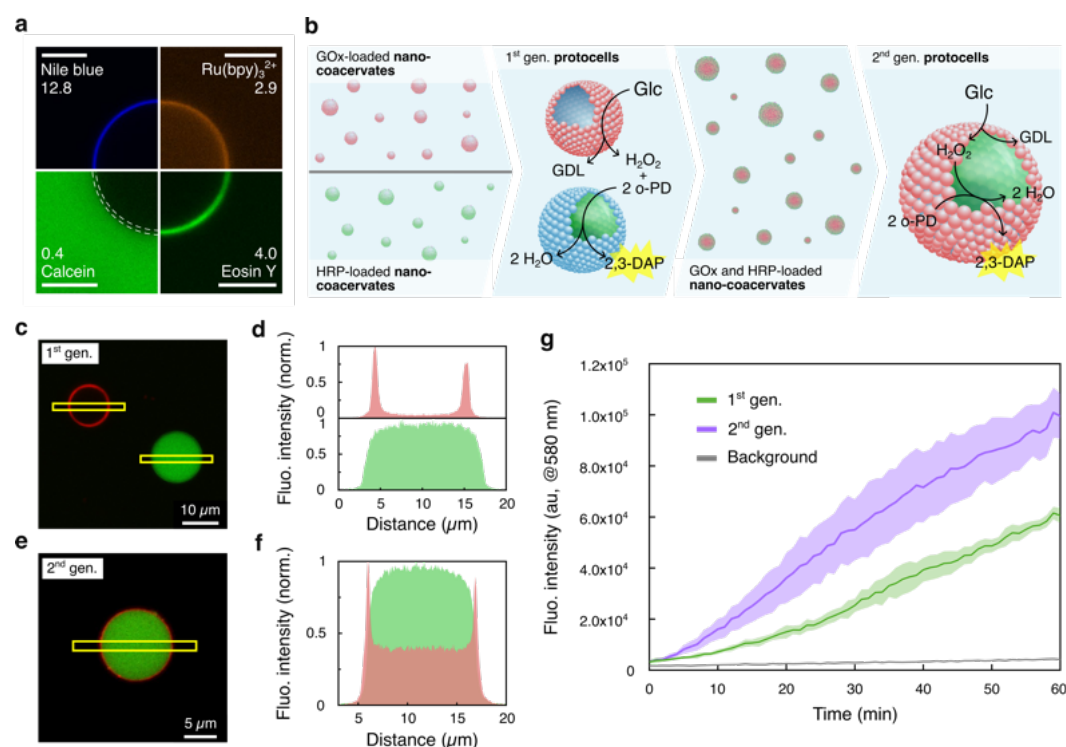


Figure 2.7 Mechanical energy-mediated evolution of a protocell consortium. **a**, Confocal laser scanning microscopy images showing fluorescent molecule uptake by coacervate vesicles (1:4 PDMAEMA/PAA, 10 mM). Membrane equilibrium distribution coefficients are shown next to each dye name. The dashed white lines in the calcein image indicate the vesicle membrane. Scale bars = 10 μm . **b**, Coacervate vesicle preparation and evolution schematic. Initial stage: populations of GOx- (red shapes) or HRP-containing (green shapes) PDMAEMA/PAA nano-coacervates (10 mM, 1:4 ratio) in separate environments. Impulsive force creates two vesicle populations containing either GOx (red, mostly present in membrane) or HRP (green, mostly present in lumen), which are mixed in 1:1 ratio to form the initial binary protocell consortium (1st generation). After vesicle disassembly, the enzymes randomly distribute in both nano-coacervate populations. Application of impulsive force creates a 2nd generation of vesicles containing both enzymes. The reaction scheme shows the H_2O_2 role as signaling molecule between enzymes. **c**, Confocal laser scanning microscopy image of 1st generation coacervate vesicles showing enzyme distribution: RITC-tagged GOx (red) localizes in the membrane, while FITC-tagged HRP (green) concentrates in aqueous lumen. **d**, Fluorescence line profile plots along the yellow areas in **c** demonstrating selective enzyme encapsulation. **e**, Confocal laser scanning microscopy image showing enzyme distribution in a single 2nd generation coacervate vesicle. **f**, Fluorescence line profile plots along the yellow area in **e**. **g**, Time-dependent fluorescence intensity ($\lambda_{\text{em}} = 580 \text{ nm}$) showing 2,3-DAP production in 1st generation (green plot, initial velocity = $6 \pm 1 \text{ I}_{\text{fluo}} \text{ s}^{-1}$) and 2nd generation (purple plot, initial velocity = $22 \pm 8 \text{ I}_{\text{fluo}} \text{ s}^{-1}$) coacervate vesicles against a hydrogel background (grey plot). Error bands show standard deviation ($n = 3$).

The two populations of GOx- or HRP-containing protocells were then mixed together in a Petri dish. Confocal microscopy confirmed the presence of these separate protocell populations (Figure 2.7b, 1st gen.). As expected for a dissipative system, they disassembled over seven days into enzyme-containing nano-coacervates (Figure 2.7b, step III). Reapplication of impulsive force produced a 2nd generation of protocells in which both enzymes coexisted within the same vesicle while maintaining their characteristic spatial segregation: GOx in the membrane and HRP in the lumen (Figure 2.7e,f). This cycle illustrates how mechanical energy can not only assemble protocells but also drive their evolution into hierarchically organized structures.

To study the chemical communication between the binary population of GOx- or HRP-containing protocells (1st generation) and monitor their evolution into metabolically enhanced 2nd-generation protocells, we embedded both generations in an agarose hydrogel matrix within a cuvette for kinetic fluorescence assays. The hydrogel slowed substrate diffusion, enabling a clear resolution of reactivity differences between protocell generations (Figure S14).

To monitor chemical communication and enzymatic enhancement, the 1st- and 2nd-generation protocells were embedded in agarose hydrogel for kinetic fluorescence assays (Figure S14). In the 1st-generation system, glucose oxidation by the GOx-containing protocells produced H₂O₂, which diffused to HRP-containing protocells, triggering o-phenylenediamine (o-PD) oxidation to fluorescent 2,3-diaminophenazine (2,3-DAP) (Figure 2.7b). Under identical conditions, the 2nd-generation protocells exhibited an initial 2,3-DAP formation rate more than three times higher (Figure 2.7g). The enhanced activity arises from their internal “all-in-one” architecture: GOx positioned in the membrane provides efficient H₂O₂ transfer directly toward the HRP-loaded lumen. Measurements of pH decrease, associated solely with glucono-δ-lactone hydrolysis and independent of H₂O₂ diffusion, were comparable in both systems, confirming that the overall rate enhancement originates specifically from improved signaling efficiency rather than intrinsic changes in GOx activity (Figure S14).

2.5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this chapter, I have shown that mechanical energy can act as an effective driver for the DSA of coacervate vesicles. By applying an impulsive force to nano-coacervates formed near the coacervation phase boundary, it was possible to induce their transient reorganization into hollow vesicles with micrometric dimensions. These structures remained stable only under continuous energy dissipation and gradually returned to their nanodroplet state in its absence, highlighting the non-equilibrium nature of the assembly process. Importantly, the resulting vesicles retained typical features of complex coacervates, such as the ability to concentrate specific molecules, while also exhibiting new properties, including spatial segregation between membrane and lumen.

The formation of mechanically assembled coacervate vesicles was not limited to the PDMAEMA/PAA system. Similar behavior was observed in other binary polyelectrolyte combinations, including both synthetic and bio-derived polymers. This generality suggests that the mechanism identified here is not restricted to a specific chemistry and may occur in systems featuring an imbalance of surface charges and sufficient polymer mobility. The combination of DSA and selective molecular uptake enabled the construction of protocells with basic metabolic and communication capabilities. By preparing two distinct protocell populations containing either GOx or HRP, and by exploiting their reversible dissipative behavior, it was possible to generate an evolved protocell generation in which both enzymes coexisted yet remained spatially organized. This rearrangement significantly improved the efficiency of the enzymatic cascade reaction, demonstrating how mechanical energy can drive the progression from simple, separated units to a more integrated and functionally enhanced protocellular system.

From a broader perspective within this thesis, these findings establish mechanical energy as a viable and versatile input for directing non-equilibrium assembly processes. This expands the toolkit available for designing adaptive soft-matter systems and provides a foundation for future studies aimed at integrating more complex functionality into mechanically controlled protocells. The implications span multiple

fields: for example, in biotechnology, this mechanism could revolutionize drug delivery by enabling the direct self-assembly of DNA or RNA-based drugs into vesicular carriers through simple vial agitation, potentially eliminating the need for additional carriers. Our findings bridge a crucial gap in DSA research while simultaneously advancing frontiers in protocell engineering, supramolecular chemistry, soft matter chemistry and physics, micro-bioreactor technologies, drug delivery, and origin-of-life research.

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2.7 APPENDICES

2.7.1 Materials and methods

All solvents were purchased in analytical grade from Sigma-Aldrich and used without further purification. Milli-Q water was obtained from a Millipore Milli-Q Plus 185 apparatus and presented a resistivity of 18.2 MΩcm. All reagents were used as received unless otherwise stated. Poly(acrylic acid sodium salt) (PAA; 2.1 kDa), poly(diallyldimethylammonium chloride) solution (< 100 kDa; PDDA), deoxyribonucleic acid from salmon sperm (dsDNA; low molecular weight), poli-L-lysine hydrobromide (PLys; 15 – 30 kDa), glucose oxidase (GOx) (252100 U mg⁻¹), horseradish peroxidase (HRP) (172 U mg⁻¹), fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), o-phenyldiamine (o-PD), α-D-glucose (Glc), propanethiol, sodium hydroxide, carbon disulphide, tosyl chloride, 4,4-azobis(4-cyanovaleric acid) (ACVA), 2,2'-azobis(2-metilpropionitrile) (AIBN), N,N-(dimethylamino)ethyl methacrylate (DMAEMA), acrylic acid (AA), fluorescein O-methacrylate, multipurpose agarose, and PBS Tablets were purchased from Sigma-Aldrich. Methacryloxyethyl thiocarbamoyl rhodamine B was purchased from Polysciences.

¹H and ¹³C NMR spectra were recorded on a Jeol GX-400 MHz spectrometer. ¹H NMR spectra are reported as δ in units of parts per million (ppm) relative to chloroform (δ 7.26, s) and water (δ 4.79, s). The number of protons (n) for a given resonance is indicated as nH and was based on spectral integration values. ¹³C NMR spectra are reported as δ in units of parts per million (ppm) relative to CDCl₃ (δ 77.16, t).

Dynamic light scattering and zeta potential measurements were performed on a Zetasizer Nano series instrument (Malvern Instruments, UK).

UV-vis spectroscopy was conducted using a Cary 5000 UV-Vis-NIR spectrophotometer, in the wavelength range of 175–900 nm. The instrument was equipped with tungsten-halogen lamps for the visible range and deuterium arc lamps for the UV range.

Attenuated total reflectance Fourier transform infrared (ATR-FT-IR) spectra were recorded using a Shimadzu IR Affinity-1S Fourier Transform Infrared spectrophotometer. The data were processed and analyzed using LabSolutions IR software.

A Seven Compact digital pH-meter by Mettler Toledo was used for pH determinations. The pH electrode was an InLab Semi-Micro glass electrode. The pH meter was calibrated before each set of measurements using standard buffer solutions at pH 4.01, 7.00, and 10.01, ensuring accurate readings across the relevant pH range.

Fluorescence spectroscopy measurements were performed on an Edinburgh Instruments FS5 spectrofluorometer using a 150 W CW Ozone-free xenon arc lamp as a source and a Photomultiplier R928P (spectral coverage 200 nm – 900 nm, cooled and stabilized) as the detector.

The degree of polymerization (DP) of the polymers synthesized in this work was determined from the ^1H NMR spectra of the polymerization solution taken before and after the polymerization reaction. Dimethylformamide (DMF) was used as an internal standard.

The determination of the molecular weight of the synthesized polymers was performed with a Malvern OMNISEC chromatography system configured with a quadruple detector system (GPC). The OMNISEC REVEAL detector system consisted of a differential refractometer ($\lambda = 640$ nm), a UV-vis diode array spectrometer ($\lambda = 190$ –900 nm), a light scattering detector with right angle light scattering (RALS) operating at 90° and low angle light scattering (LALS) at 7° ($\lambda = 640$ nm), and a differential viscometer comprising four capillaries, a delay column, and two pressure transducers (DP and IP). The detectors were arranged in the following order: RI – UV – RALS –

LALS – viscosimeter. All detectors were calibrated with pullulan and dextran in an aqueous mobile phase. The OMNISEC RESOLVE system included pre-installed mobile phase pumps, an autosampler with a 300 μ L injection loop, a degasser, and SEC columns. OMNISEC v11.41 software was used for SEC data acquisition and analysis. A 0.2 μ m cellulose acetate filter was placed between the columns and the detectors. The eluent was filtered using a 0.1 μ m Omnipore PTFE filter. The delays between the detectors and the band broadening parameters were automatically calculated by the OMNISEC software using pullulan as the narrow standard. The stationary phase consisted of a Tosoh TSKgel G5000 cationic aqueous solvent column (300 mm length, 7.8 mm ID) and a Tosoh TSKgel G3000 cationic aqueous solvent column (300 mm length, 7.8 mm ID) connected in series. The two chromatography columns were preceded by a TSKgel PWXL guard column (40 mm length, 6 mm ID). Isocratic elution was performed with a flow rate of 0.5 mL/min, and 50 μ L of sample at a concentration of 3 mg/mL was injected.

Optical and fluorescence microscopy was performed on an Axio Observer 7 (Zeiss) microscope at 20 $\times$ and 40 $\times$ magnification. The microscope was equipped with an Orca Flash 4.0 V3 CMOS camera (Hamamatsu) and a motorized XYZ sample stage. The brightfield images were obtained using a transmitted light LED lamp. Images were analyzed using the latest version of ImageJ software.

Confocal laser scanning microscopy images were obtained on a FV3000 (Olympus) confocal laser scanning microscope. The system was equipped with 4 excitation laser lines: 405, 488, 561, and 640 nm (Coherent), a galvanometric scanning head, and 4 spectral detectors, allowing for simultaneous imaging of up to 4 fluorescence channels. Brightfield images were acquired using a scanning laser line and a transmitted light detector. The inverted microscope body (Olympus IX 83) included a motorized automated sample stage for multi-area tiled imaging. The following objectives were used: UPLXAPO 20 $\times$ /0.8 NA (Olympus), and an oil objective UPLXAPO 60 $\times$ /1.42 NA (Olympus). Images were taken using excitation wavelengths of 405 nm (o-PD), 488 nm (FITC), and 561 nm (RITC) and emission wavelengths of 550-625 nm, 500-

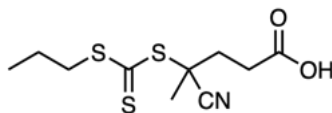
550 nm, and 575-650 nm, respectively. Images were analyzed using the latest version of ImageJ software.

For the scanning electron cryo-microscopy (Cryo-SEM) analysis, the liquid samples were placed in the holders of a custom-made ZEISS shuttle and transferred to a Leo Crossbeam 1540XB FIB-SEM (ZEISS Microscopy, Oberkochen, Germany) using a QUORUM PP3010Z cryo-stage with a special transfer system. The samples were rapidly frozen at -190 °C, followed by sublimation at -90 °C for 20 minutes to remove surface water. Subsequently, the samples were sputtered with platinum at a current of 10 mA for 30 s. To obtain vesicle cross-sections, the frozen samples were fractured and cut open with a scalpel before sublimation inside the cryo-stage. The temperature was maintained at -175 °C throughout the imaging process. Images were captured using Zeiss SmartSEM software and analyzed using the latest version of ImageJ software.

SANS experiments were conducted on the SANS2d and Zoom instruments at the ISIS Neutron Source (STFC Rutherford Appleton Laboratory) in Oxfordshire, UK. For SANS2d, a simultaneous Q-range of 0.005 – 1.0 Å⁻¹ was achieved utilizing an incident wavelength range of 1.75 – 16.5 Å. The beam is centered on the 96.5 × 96.5 cm² detector, with a fixed distance of 8 m between the sample and the detector. The beam size was 8 mm × 8 mm. For Zoom, a simultaneous Q-range of 0.005 – 1.0 Å⁻¹ was achieved utilizing an incident wavelength range of 1.75 – 16.5 Å. The beam is centered on the 96.5 × 96.5 cm² detector, with a fixed distance of 8 m between the sample and the detector. The beam size was 6 mm × 8 mm. The solutions were contained in Hellma quartz spectrophotometer cuvettes with a path length of 2 mm and were maintained at a temperature of 25 ± 1°C. The scattering from the empty cell and solvent (D₂O) was subtracted from the raw data. The data were reduced using Mantid and analyzed with the latest version of SasView.

2.7.2 Synthesis of molecules and polymers

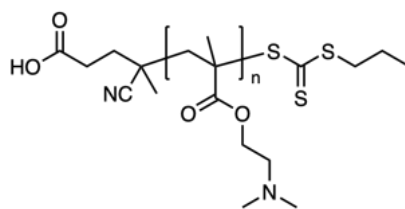
4-Cyano-4-[(propylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CPP)



CPP was synthesized following a previously published procedure, which was slightly modified.⁵⁷ Propanethiol (0.076 g, 1.0 mmol) was dissolved in a 1:1 mixture of acetone and water (8 mL) with sodium hydroxide (0.10 g, 2.0 mmol). After cooling to 0°C, carbon disulfide (0.092 g, 73 μ L, 1.2 mmol) was added dropwise while stirring. After 2 hrs, the reaction mixture was cooled to -5°C with an acetone/ice bath, and a tosyl chloride (0.23 g, 1.2 mmol) solution in acetone (2 mL) was added dropwise, and the reaction was stirred for a further 1 hr at room temperature. The reaction mixture was acidified to pH 2 with HCl (1 M) and extracted with ethyl acetate (2 x 20 mL). The solution was then concentrated to *ca.* 10 mL by rotary evaporation, and ACVA (0.56 g, 2.0 mmol) was added, and the mixture was stirred under reflux at 80°C overnight. The crude was purified by column chromatography without workup, over silica, using ethyl acetate/hexane (1:3) + 1% (v/v) acetic acid as the eluent. The product was a yellow oil (0.20 g, 0.72 mmol, yield = 72 wt%).

¹H NMR (400 MHz, CDCl₃): δ (ppm) = 3.32 (t, J = 7.2 Hz, 2H, SCH₂), 2.74 – 2.61 (m, 2H, CH₂COOH), 2.60 – 2.33 (m, 2H, CH₂CH₂COOH), 1.88 (s, 3H, CH₃), 1.74 (h, J = 7.3 Hz, 2H, CH₃CH₂), 1.02 (t, J = 7.3 Hz, 3H, CH₃CH₂).

¹³C NMR (101 MHz, CDCl₃): δ (ppm) = 217.0, 176.7, 119.0, 46.4, 39.0, 33.7, 29.6, 25.0, 21.4, 13.6.

Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA)

For 20 kDa PDMAEMA. AIBN was freshly recrystallized from methanol, DMAEMA monomer was purified from the inhibitor through a basic alumina column. DMAEMA (0.486 g, 3.09 mmol), AIBN (1.31 mg, 8.00 μmol) and CPP (11.1 mg, 40.0 μmol) were dissolved in 1 mL of 1,4-dioxane in a Schlenk tube equipped with a stirrer bar. DMF (0.1 mL) was added as an internal standard to calculate the degree of polymerization. After sealing the tube, the solution was degassed with four freeze-pump-thaw cycles and refilled with argon for 30 min. Polymerization was carried out at 65°C for 14 hrs. The polymer was isolated by precipitation from cold hexane (50 mL), and subsequently it was lyophilized to obtain a light-yellow solid in 70 wt% yield. ^1H NMR (400 MHz, CDCl_3): DP = 80%. GPC: 0.1 M NaNO_3 at pH 2.6 ($\text{CH}_3\text{CO}_2\text{H}$); M_n (GPC) = 19,677 gmol^{-1} , D (GPC) = 1.128, calculated dn/dc = 0.192.

The same procedure has been reproduced for all the different PDMAEMA with different molecular weights, varying the amount of CPP and AIBN:

10 kDa: 80% yield. ^1H NMR (400 MHz, CDCl_3): DP = 90%. GPC: 0.1 M NaNO_3 at pH 2.6 ($\text{CH}_3\text{CO}_2\text{H}$); M_n (GPC) = 13,701 gmol^{-1} , M_w (GPC) = 16,348 gmol^{-1} , D (GPC) = 1.193.

40 kDa: 65% yield. ^1H NMR (400 MHz, CDCl_3): DP = 75%. GPC: 0.1 M NaNO_3 at pH 2.6 ($\text{CH}_3\text{CO}_2\text{H}$); M_n (GPC) = 35,268 gmol^{-1} , M_w (GPC) = 41,469 gmol^{-1} , D (GPC) = 1.176.

70-80 kDa: 50% yield. ^1H NMR (400 MHz, CDCl_3): DP = 70%. GPC: 0.1 M NaNO_3 at pH 2.6 ($\text{CH}_3\text{CO}_2\text{H}$); M_n (GPC) = 70,327 gmol^{-1} , M_w (GPC) = 87,910 gmol^{-1} , D (GPC) = 1.250.

2 | MECHANICALLY DRIVEN DISSIPATIVE SELF-ASSEMBLY OF COACERVATE VESICLES

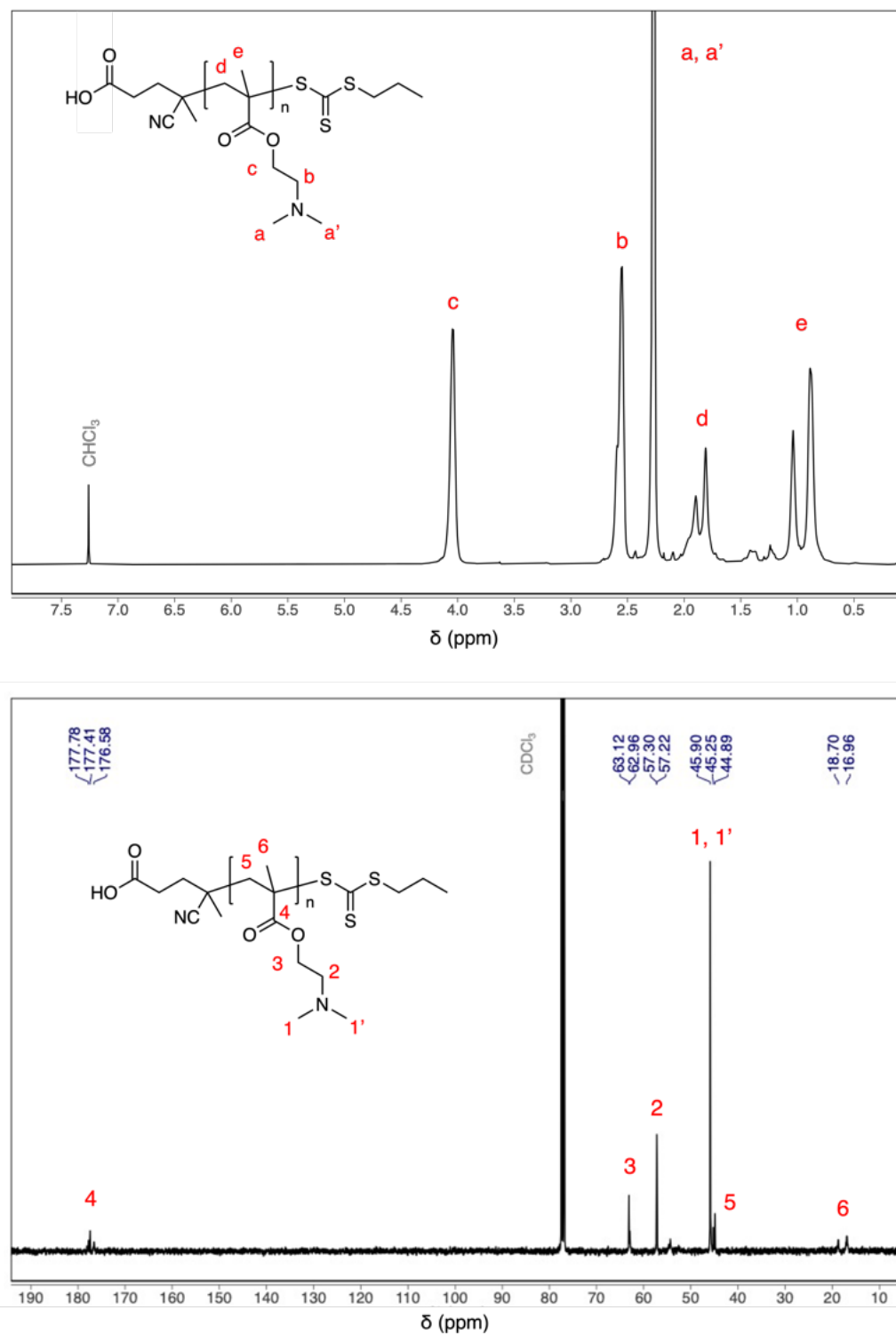


Figure S1 Top: ^1H NMR spectrum of 20 kDa PDMAEMA acquired in CDCl_3 and referenced against residual CHCl_3 . Bottom: proton decoupled ^{13}C NMR spectrum of PDMAEMA acquired in CDCl_3 and referenced against residual CDCl_3 .

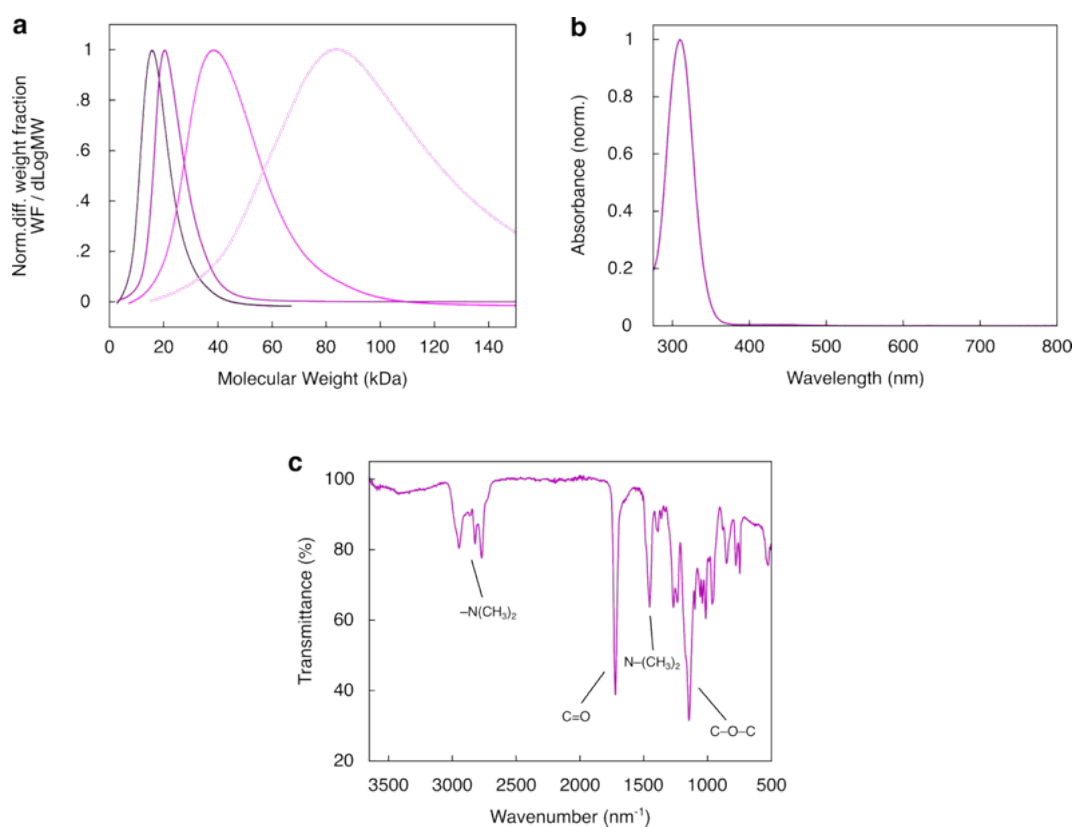
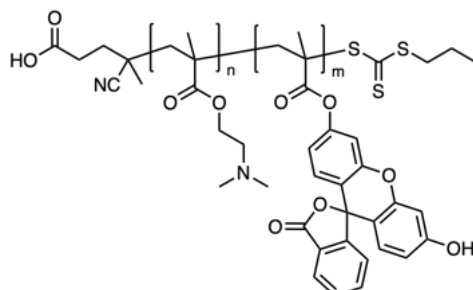


Figure S2 Characterization of PDMAEMA. **a**, GPC molar mass distribution of the different polymers. **b**, Normalized UV-vis spectrum acquired in Milli-Q water at a concentration of 1 mg mL⁻¹. **c**, ATR-FT-IR spectra of PDMAEMA.

**Poly(2-(dimethylamino)ethylmethacrylate)-co-fluorescein-O-methacrylate
(FITC-PDMAEMA)**



AIBN was freshly recrystallized from methanol, DMAEMA monomer was purified from the inhibitor through a basic alumina column. DMAEMA (0.499 g, 3.17 mmol), fluorescein O-methacrylate (0.51 mg, 1.30 μ mol), AIBN (1.31 mg, 8.00 μ mol) and CPP (11.1 mg, 40.0 μ mol) were dissolved in 1 mL of 1,4-dioxane in a Schlenk tube equipped with a stirrer bar. DMF (0.1 mL) was added as an internal standard to calculate the degree of polymerization. The tube was sealed, the solution was degassed with four freeze-pump-thaw cycles and purged with argon for 30 min. Polymerization was carried out at 65°C for 14 hrs. The polymer was isolated by precipitation from cold hexane (50 mL) and lyophilized to obtain a light-yellow solid in 77 wt% yield. ^1H NMR (400 MHz, CDCl_3): DP = 85%. GPC: 0.1 M NaNO_3 at pH 2.6 ($\text{CH}_3\text{CO}_2\text{H}$); M_n = 20,748 g mol^{-1} , Đ = 1.161, calculated dn/dc = 0.198.

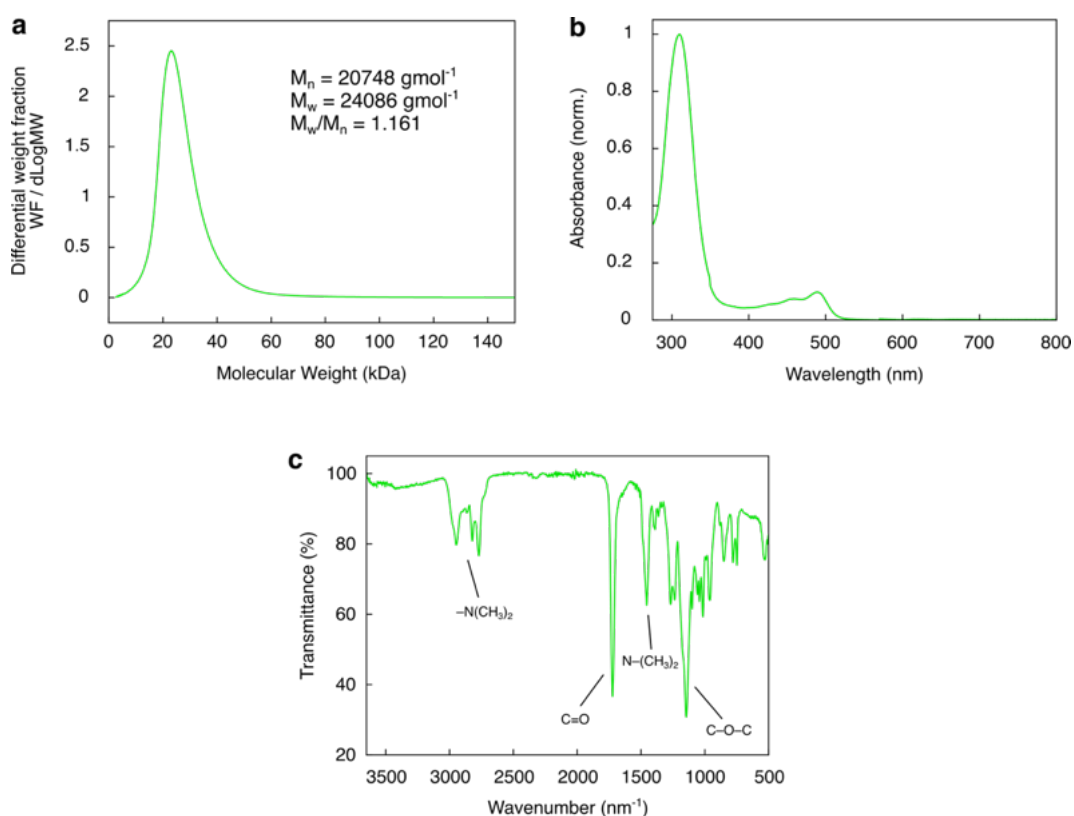
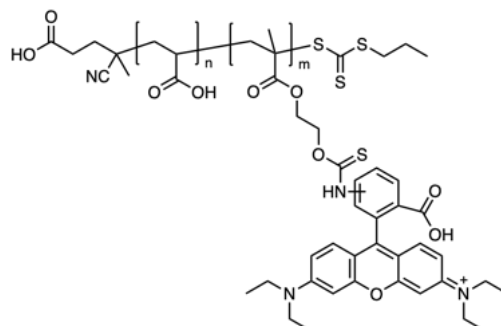


Figure S3 Characterization of FITC-PDMAEMA. **a**, GPC molar mass distribution. **b**, Normalized UV-vis spectrum acquired in Milli-Q water at a concentration of 1 mg mL⁻¹. In contrast with the UV-vis spectrum of PDMAEMA, the UV-vis spectrum of FITC-PDMAEMA shows clear presence of FITC. The degree of labelling was 0.02, meaning that not all polymer chains contain an FITC moiety. This degree of labelling was sufficient for all our experiments. **c**, ATR-FT-IR spectra of FITC-PDMAEMA.

Synthesis of poly(acrylic acid)-co-rhodamine B methacrylate sodium salt (RITC-PAA)



Acrylic acid (0.499 g, 6.92 mmol), methacryloxyethyl thiocarbamoyl rhodamine B (0.93 mg, 1.4 μ mol), ACVA (2.24 mg, 8.00 μ mol) and CPP (23.5 mg, 80.0 μ mol) were dissolved in 1 mL of 1,4-dioxane in a Schlenk tube equipped with a stirrer bar. DMF (0.1 mL) was added as an internal standard to calculate the degree of polymerization. The tube was sealed, the solution was degassed with three freeze-pump-thaw cycles and purged with argon for 30 min. Polymerization was carried out at 70°C for 2 hrs. The polymer was isolated by precipitation from cold diethyl ether (50 mL). To form the sodium salt, after elimination of the solvent, the polymer has been dissolved in Milli-Q water and the pH has been adjusted to 8.5 by adding NaOH 0.5 M aliquots. The resulting polymer has been dialyzed against water and lyophilized to obtain a pink solid in 20 wt% total yield. ^1H NMR (400 MHz, D_2O): DP = 36%, calculated molecular weight = 3,208 g mol^{-1} . GPC: 0.15 M PBS; M_n = 4,358 g mol^{-1} , $\bar{D}$ = 1.068, calculated dn/dC = 0.159.

For the GPC analysis, the stationary phase consisted of two Malvern A6000M aqueous solvent columns (300 mm length, 8 mm ID) and an A2500 column (300 mm length, 8 mm ID) connected in series. Isocratic elution was performed with a flow rate of 0.6 mL/min, and 150 μ L of sample at a concentration of 4 mg/mL was injected.

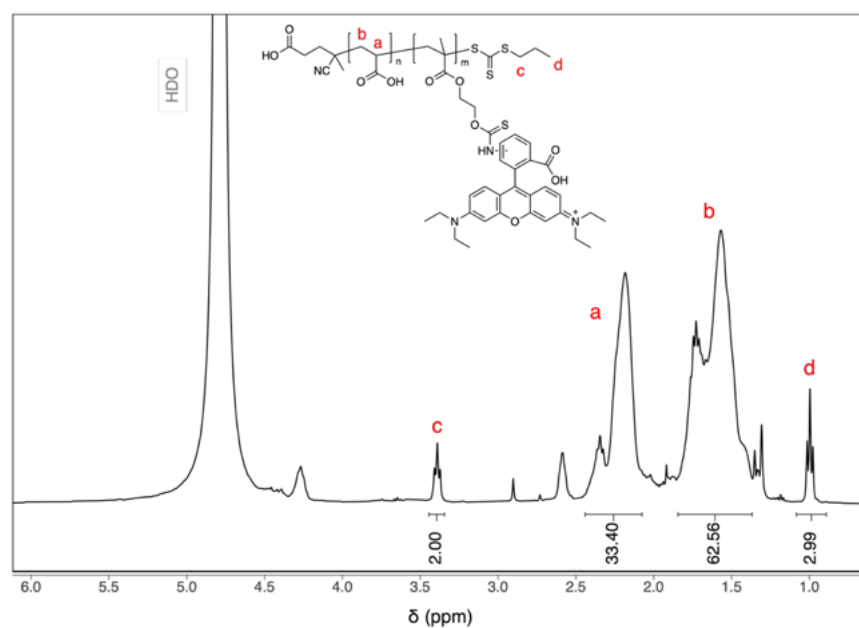


Figure S4 ^1H NMR spectrum of RITC-PAA acquired in D_2O and referenced against residual HDO. Peaks corresponding to the CPP RAFT agent (c, d) were integrated to calculate the polymer molecular weight. The ^{13}C NMR spectrum could not be obtained due to two factors: the polymer's poor solubility in common deuterated organic solvents, and the absence of detectable signals in D_2O . The latter was likely caused by rapid ion-exchange dynamics and broadening effects typical of polyelectrolytes.

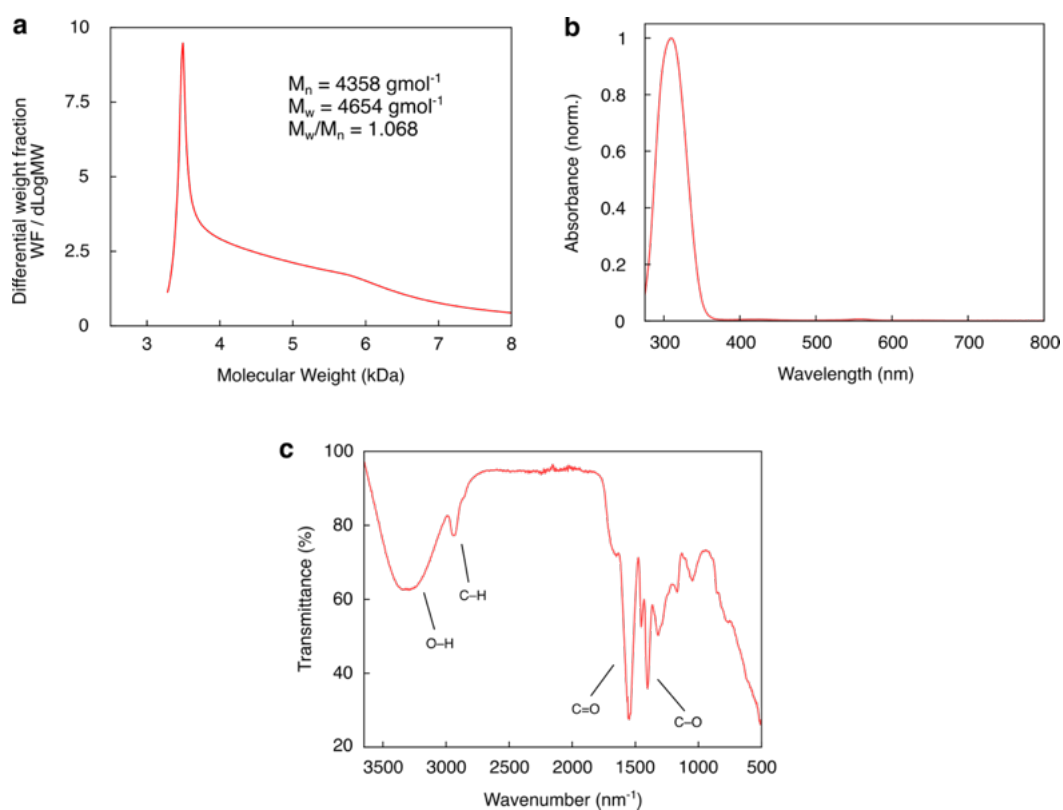


Figure S5 Characterization of RITC-PAA. **a**, GPC molar mass distribution. **b**, Normalized UV-vis spectrum acquired in Milli-Q water at a concentration of 1 mg mL^{-1} . The UV-vis spectrum shows presence of RITC. The degree of labelling was 0.0004, meaning that not all polymer chains contain an RITC moiety. This degree of labelling was sufficient for all our experiments. **c**, ATR-FT-IR spectra of RITC-PAA.

2.7.3 Experimental

A1 – Generation of phase diagrams. To obtain the phase diagram for the different pairs of polyelectrolytes, the concentration of the starting solution for each polymer was fixed at 1 mM, 5 mM, 10 mM, 20 mM, or 50 mM, the pH was adjusted to 6.5, and the mixing ratio between polycation and polyanion was systematically varied. The final volume of the solution was always 400 μ L. To fabricate the coacervate vesicles, the general procedure outlined in the experimental section was followed. The formation of the coacervate vesicles was confirmed via optical microscopy both immediately after shaking and 5 minutes later. To further assess the self-assembly of the vesicles, the samples were also analyzed using cross-polarized microscopy. Properly formed coacervate vesicles showed a characteristic birefringence.

A2 – Preparation of coacervate vesicles. Stock solutions of each polymer were prepared in Milli-Q water. For molarity calculations, we referred to the molecular weights (Mw) of the monomers (DMAEMA: 157.21 g mol⁻¹, AA(Na salt): 94.05 g mol⁻¹, DDA chloride: 161.67 g mol⁻¹, average nucleobase: 303.70 g mol⁻¹, PLys: 223.12 g mol⁻¹). The pH of the solutions was adjusted to 6.5 by the addition of either HCl (0.5 M) or NaOH (0.5 M).

For the preparation of coacervate vesicles using a 1:4 PDMAEMA/PAA molar, 100 μ L of 10 mM PDMAEMA stock solution and 400 μ L of 10 mM PAA stock solution were combined in a 1.75 mL glass vial. The solution looked clear. To form the coacervate vesicles, the resulting solution was vigorously shaken by hand for 30 s with a hand gesture that is identical to that used to lower the temperature of a thermometer, thereby producing strong shockwaves in the liquid. For instance, a vigorous stirring with a stirrer-bar or sonication failed to produce coacervate vesicles, whereas vortexing (which combines shear and impact forces) generated only a negligible number of coacervate vesicles (Figure S9).

The same method was used to generate all the different types of coacervate vesicles described in this work, with the only difference that the proper polymer solutions

and/or molar ratios were used instead of a 1:4 PDMAEMA/PAA (10 mM) molar ratio. All microscopy images were acquired from vesicles settled on the glass surface. Upon contact with glass, coacervate vesicles maintain their structural integrity, in contrast to coacervate microdroplets, which rapidly wet the glass surface and lose their spherical morphology.

A3 – Small-angle neutron scattering experiments. Samples for SANS analysis were prepared by mixing the appropriate volumes of 10 mM solutions of PDMAEMA and PAA in D₂O. These solutions were filtered through 0.22 µm nylon filters (Artiglass). The pD (pH in D₂O) was adjusted to 6.5 using 0.5 M DCl, and measurements were conducted using a digital pH-meter equipped with a glass electrode. pH-meter readings in D₂O were corrected using the formula $pD = pH + 0.40$.⁵⁸

Samples were studied both before and after 30 s of manual shaking and prepared by directly mixing the solutions of the two polymers in a 1.75 mL glass vial. Vesicle samples were prepared from the resulting solutions, which were vigorously shaken by hand for 30, as described in the methods section. The solutions were then transferred to Hellma quartz spectrophotometer cuvettes with a path length of 2 mm for SANS measurements. The raw scattering data were reduced using Mantid software, where the scattering from the solvent (D₂O) and the empty cell was subtracted.

For data analysis in SasView, the scattering profiles of the coacervate microdroplet samples were fitted using a correlation length model, as previously reported, to determine the overlapping of polymer chains in the complex coacervate phase.⁴⁹ The scattering intensity $I(q)$ is calculated as:

$$I(q) = \frac{A}{q^n} + \frac{C}{1+(q\xi)^m} + background \quad (\text{Equation S1})$$

where the two multiplicative factors A and C , and the two exponents n (Porod) and m (Lorentz) are used as fitting parameters. The ξ parameter is a correlation length for the polymer chains. The typical correlation length ξ of complex coacervates is a few nanometers, indicating polymer chain overlap.

Table S1: SANS fitting parameters for 1:1 PDMAEMA/PAA (10 mM) molar ratio coacervate microdroplets before (a) and after application of impulsive force (b), and for 10 mM solution of PDMAEMA in D₂O.

	A	C	$backgr$ (10 ³)	ξ (nm)	n	m	χ^2
a	$5.55 \cdot 10^{-12}$	0.08	1.10	2.17 ± 0.04	4.00	2.37	0.70
b	$5.70 \cdot 10^{-8}$	0.07	1.93	2.55 ± 0.06	3.05	2.17	0.78
PDMAEMA	$2.32 \cdot 10^{-10}$	0.08	3.62	2.12 ± 0.04	3.57	2.21	1.49

For the 1:4 molar ratio PDMAEMA/PAA samples, a core-shell ellipsoid model was applied to fit the data.

The intensity is given by:

$$I(q, \alpha) = \frac{scale}{V} F^2(q, \alpha) + background \quad (\text{Equation S2})$$

where a is the orientation angle of the ellipsoid, and where:

$$F(q, \alpha) = f(q, R_e, R_e \cdot x_{core}, \alpha) + f(q, R_e + t_{shell}, R_e \cdot x_{core} + t_{shell} \cdot x_{polarshell}, \alpha)$$

$$(\text{Equation S3})$$

And:

$$f(q, R_e, R_p, \alpha) = \frac{3\Delta\rho V(\sin(qr) - qr \cos(qr))}{(qr)^3} \quad (\text{Equation S4})$$

For:

$$r = [R_e^2 \sin^2 \alpha + R_p^2 \cos^2 \alpha]^{1/2} \quad (\text{Equation S5})$$

where V is the volume of the ellipsoid with R_e as the equatorial radius, and $R_p = R_e \cdot x_{core}$ as the polar radius. t_{shell} is the thickness of the shell at the equator, and $\Delta\rho$ is the scattering length density difference, *i.e.*, $\Delta\rho = (\rho_{shell} - \rho_{solvent})$ or $(\rho_{core} - \rho_{solvent})$.

Table S2: SANS fitting parameters for the 1:4 PDMAEMA/PAA (10 mM) molar ratio nano-coacervates before (a) and after application of impulsive force (b).

	<i>Scale</i>	<i>backgr</i> (10 ³)	R_e (nm)	x_{core}	t_{shell} (nm)	ρ_{shell} (10 ⁶ Å ⁻²)	ρ_{core} (10 ⁶ Å ⁻²)	χ^2
a	0.10	3.77	35.9 ± 4.6	0.75 ± 0.13	61.8 ± 3.5	6.33	5.84	1.02
b	0.12	1.80	48.0 ± 1.6	0.53 ± 0.02	57.2 ± 2.9	6.33	5.84	0.88

Fitting parameters were optimized to achieve the best agreement between the models and the experimental data (χ^2).

A4 – Reversible generation of coacervate vesicles. To study the disassembly and the regeneration of the coacervate vesicles, coacervate vesicles were prepared as described in the methods section, and were subsequently monitored over time at room

temperature using brightfield microscopy. The size and count of vesicles and coacervate microdroplets were automatically measured and analyzed using the built-in ZEN Blue software on the ZEISS Axio Observer microscope. This software enabled the precise determination of size distribution, and the quantification of vesicle count at each time point.

To investigate the reversible formation-disassembly process of coacervate vesicles, the same methodology was followed, with the only difference that the disassembly process was accelerated using a ThermoFisher tube revolver in agitation mode. After complete dissolution (24 hrs), samples were re-shaken manually for 30 s to reform the coacervate vesicles, and the analysis process using brightfield microscopy was repeated.

A5 – Fluorescence recovery after photobleaching (FRAP). Coacervate vesicles were formed utilizing the general procedure described in the methods section, with the only difference that FITC-PDMAEMA was used in place of PDMAEMA. Coacervate vesicles were formed at a 1:6 molar ratio between FITC-PDMAEMA and PAA (10 mM), whereas complex coacervate microdroplets were formed at a molar ratio of 1:1. The two samples were used to perform FRAP experiments on an Olympus FV3000 confocal laser scanning microscope utilizing a 60x oil immersion objective lens. For both samples excitation was performed with a 488 nm laser and the emission was recorded between 500 – 550 nm. Fluorescence recovery was recorded for 10 minutes with an acquisition rate of one frame every 30 s. To obtain a pre-bleaching reference, two images were taken before bleaching, always with an acquisition rate one frame every 30 s. Bleaching was performed using a 488 nm laser at 50% intensity for 2 s in a circular area of approximately 1 μm in radius, with a scanning speed of 2 $\mu\text{s}/\text{pixel}$. FRAP analysis was carried out using the latest version of ImageJ software. The fluorescence intensity in the selected area was normalized with the intensity in an area on the opposite side of the vesicle or microdroplet to account for any overall photobleaching effects, as shown in the formula:

$$I_{norm}(t) = \frac{I_{bleach}(t) - I_{background}(t)}{I_{reference}(t) - I_{background}(t)} \quad \text{(Equation S3)}$$

Where, for every image at time of the experiment time lapse:

$I_{norm}(t)$ is the normalized fluorescence intensity.

$I_{bleach}(t)$ is the fluorescence intensity of the bleached region.

$I_{background}(t)$ is the fluorescence intensity of the background (outside the vesicle/microdroplet).

$I_{reference}(t)$ is the fluorescence intensity of the reference region (opposite side of the vesicle/microdroplet).

A6 – Nano-coacervates fusion based on impulsive pressure. The formation of coacervate vesicles under mechanical agitation likely results from impact-induced fusion of nanodroplets, overcoming electrostatic and osmotic repulsion. This hypothesis is because a jerking motion (*i.e.*, a sudden stop and reversal of movement) is required, suggesting that transiently high pressures are necessary.

At a 1:4 PDMAEMA:PAA molar ratio, the nanodroplets can be approximated as core-shell structures (see Figure 2d, Figure S8), where excess PAA forms a negatively charged shell with a measured zeta potential $\zeta = -44.0 \pm 6$ mV. The electrical double-layer pressure stabilizing these droplets can be estimated using the Debye-Hückel approximation:

$$P_{edl} \approx 2\varepsilon_0\varepsilon_r\kappa^2\zeta^2 \approx 300 - 1000 \text{ Pa} \quad (\text{Equation S4})$$

Where, $\varepsilon_0 = 8.85 \times 10^{-12} \text{ F m}^{-1}$ the permittivity of the free space and $\varepsilon_r = 78.5$ the dielectric constant of water, assuming the Debye constant $\kappa \sim (1.25 - 1.80) \times 10^7 \text{ m}^{-1}$.^{59,60}

Additionally, the osmotic pressure of the PAA shell, arising from confined monomers, can be estimated using the de Gennes blob scaling argument:⁶¹

$$P_{osm} \approx \frac{k_B T}{s^3} \quad (\text{Equation S5})$$

where $s \sim 15$ nm is the estimated mesh size of the PAA shell. This yields $P_{osm} \approx 1200$ Pa, giving a total repulsive pressure of:

$$P_{rep} = P_{edl} + P_{osm} \approx 1500 - 2200 \text{ Pa} \quad (\text{Equation S6})$$

To overcome this repulsion, the jerking motion applies an impact force to the liquid. For a 1 mL solution (~ 1 g) subjected to sudden motion reversal at $v \sim 5 - 10$ m s⁻¹ over $Dt \sim 50 - 100$ ms (as estimated from video recordings of vial shaking during coacervate vesicle formation), the impulse-momentum equation gives:

$$F_i \approx m \frac{2v}{\Delta t} \approx 0.1 - 0.4 \text{ N} \quad (\text{Equation S7})$$

With an estimated impact area of $A \sim 1$ cm², the impact pressure is:

$$P_i = \frac{F}{A} \approx 1000 - 4000 \text{ Pa} \quad (\text{Equation S8})$$

Notably, in the context of natural phenomena, an impulsive pressure of this order of magnitude (*i.e.*, $\sim 1000 - 2000$ Pa) may be achieved by a falling rain drop of 2 mm in diameter reaching a ground impact velocity of $3 - 5$ m s⁻¹ and with an impact time of $\sim 1 - 2$ ms.⁶² The impulsive pressure exerted on rocks from crashing ocean waves could readily reach more than **10000 Pa**, whilst it is plausible that a smaller pressure would be achieved by a gentler caressing wave.⁶³

Since P_i is comparable to P_{rep} , the impact force likely overcomes nanodroplet electrostatic repulsion, triggering their fusion into vesicles. However, the vesicles gradually disassemble over time, suggesting that the fused structures exist in a metastable dissipative state rather than a thermodynamically favored one.

A7 – Retention and uptake studies. The concentration of the solutions of guest components was fixed to 1 mg mL⁻¹, and the volume added was 5% of the volume of PDMAEMA. The uptake of the fluorescent dyes inside the vesicles was determined from confocal laser scanning microscopy images by studying the emission line profile

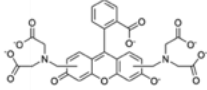
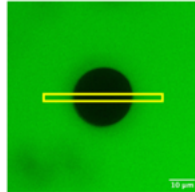
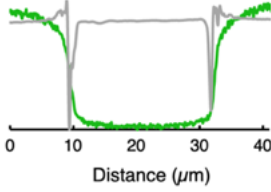
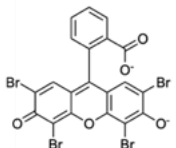
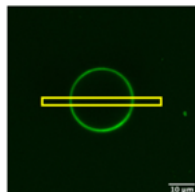
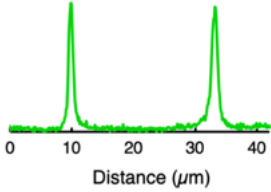
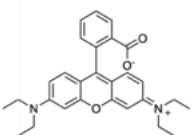
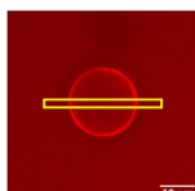
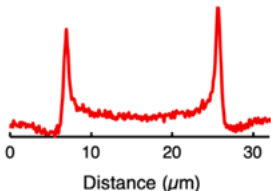
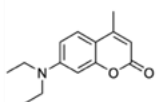
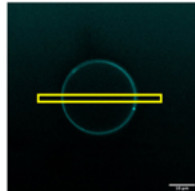
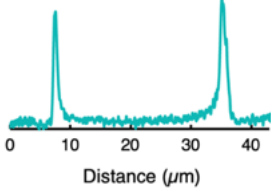
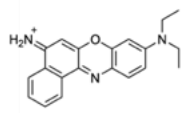
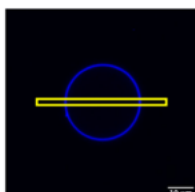
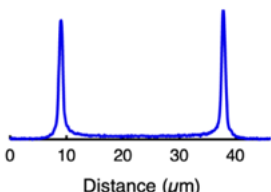
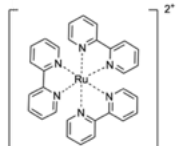
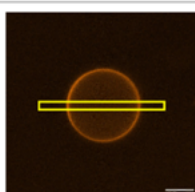
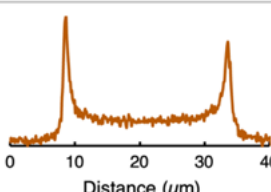
across the loaded vesicle. This also allowed for the determination of the equilibrium distribution coefficient, defined as the maximum fluorescence intensity inside the coacervate vesicle membrane and/or aqueous lumen over the averaged fluorescence intensity of the bulk aqueous phase ($I_{\text{coacervate vesicle}}/I_{\text{bulk}}$). The list of all fluorescent dyes used along with the corresponding microscope settings, is provided in **Table S3**. Brightfield images were also acquired to easily identify the membrane of non-fluorescent or low fluorescent coacervate vesicles. All images were analyzed with the last version of ImageJ software.

Table S3: Details of the fluorescence confocal laser scanning microscope settings employed in this study.

Sample	λ_{exc}	λ_{emis}	Laser int.	Sensitivity
Nile Blue	640 nm	650 – 750 nm	2.0 %	500 V
[Ru(bpy) ₃] ²⁺	488 nm	600 – 700 nm	3.0 %	500 V
Rhodamine B	561 nm	570 – 670 nm	0.05 %	500 V
Calcein	488 nm	500 – 600 nm	0.1 %	470 V
Eosin Y	488 nm	510 – 610 nm	0.1 %	500 V
7-diethylamino-4-methyl coumarin	375 nm	420 – 520 nm	6.5 %	500 V

RITC-tagged GOx	561 nm	570 – 670 nm	2.75 %	485 V
FITC-tagged HRP	488 nm	500 – 600 nm	2.5 %	485 V

Table S4: Molecular retention and uptake study of coacervate vesicles.

Dye	Charge	logD	Eq. distribution coefficient		Representative microscopy image	Fluorescence line profile (norm.)
			Mem.	Lumen		
 Calcein	-6	-15.88	0.4	0.5		
 Eosin Y	-2	0.58	4.0	1.1		
 Rhodamine B	0	-4.51	1.6	1.0		
 Coumarin 1	0	2.86	3.1	1.1		
 Nile Blue	+1	3.24	12.8	1.3		
 [Ru(bpy) ₃] ²⁺	+2	0.05 (logP)	2.9	1.4		

Molecular structures at pH 6.5, water/octanol equilibrium distribution coefficients (logD) and net charges were calculated with MarvinSketch (Chemaxon). An

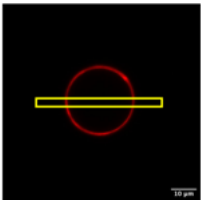
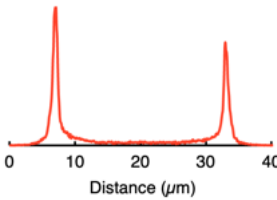
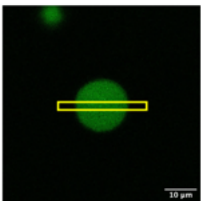
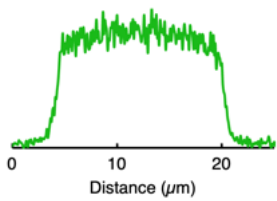
equilibrium distribution coefficient value greater than 1 indicates that the fluorescent species is sequestered inside the coacervate vesicle membrane or lumen.

A8 – Enzyme labelling. In general, 10 mg of enzyme were dissolved in 73.5 mL of Na_2CO_3 buffer (pH 8.5, 100 mM). Fluorescent tags fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) were dissolved in DMSO to yield 1 mg mL^{-1} stock solutions. 100 μL of the fluorescent tag solution was added to the enzyme aqueous solution and the mixture was stirred overnight at 4°C . The product was purified by dialysis, lyophilized and stored as a solid at -20°C .

The degree of labelling of the tagged enzymes was calculated using UV-vis spectroscopy [FITC $\epsilon_{485 \text{ nm}} = 50,400 \text{ L mol}^{-1} \text{ cm}^{-1}$, GOx $\epsilon_{280 \text{ nm}} = 3,006,000 \text{ L mol}^{-1} \text{ cm}^{-1}$, RITC $\epsilon_{559 \text{ nm}} = 62,100 \text{ L mol}^{-1} \text{ cm}^{-1}$, HRP $\epsilon_{403 \text{ nm}} = 102,000 \text{ L mol}^{-1} \text{ cm}^{-1}$] and determined to be 14.6 GOx-RITC and 2.7 for HRP-FITC.

A9 – Evolution experiments. Enzyme-loaded coacervate vesicles were prepared as described in the methods section. Separate populations of first-generation coacervate vesicles were formed, each encapsulating either RITC-tagged glucose oxidase (RITC-GOx) or FITC-tagged horseradish peroxidase (FITC-HRP). These two coacervate vesicles populations were then mixed in equal volumes (1:1 ratio) within a 1.75 mL glass vial and imaged using confocal laser scanning microscopy. After seven days, allowing for complete disassembly, the vial was re-shaken for 30 s to form a second-generation of coacervate vesicles containing both enzymes within the same micro-structure. Confocal laser scanning microscopy images were taken at this stage to confirm co-encapsulation of the two enzymes. All images were analyzed using fluorescence line intensity profile to localize the two differently tagged enzymes within the vesicle structure.

Table S5: Enzymatic retention and uptake study of coacervate vesicles.

Dye	Zeta potential (mV)	Eq. distribution coefficient		Representative microscopy image	Fluorescence line profile (norm.)
		Mem.	Lumen		
RITC-tagged GOx	-10.0 ± 0.8	16.1	1.3		
FITC-tagged HRP	$+4.5 \pm 1.0$	1.7	4.1		

A10 – Enzymatic cascade reactions. Due to the gradual sedimentation of the coacervate vesicles over time, fluorescence spectroscopy kinetics for enzyme cascade reactions were performed with the vesicles embedded on an agarose hydrogel matrix, which was immobilized on the side of a 1 mL cuvette. For this, first, a 1 wt% agarose solution was prepared by dissolving agarose powder in Milli-Q water. The mixture was heated in a glass vial at 80°C for 2 hrs, until the agarose was fully dissolved, and the solution became transparent. The agarose solution was then allowed to cool down to 50°C. For the enzyme cascade reaction experiment, 200 μL of the 50 °C agarose solution was immediately mixed with 100 μL of an aqueous suspension of coacervate vesicles (prepared from PDMAEMA 10 mM, 100 μL ; non-tagged enzymes solution 1 mg mL^{-1} , 5 μL ; PAA 10 mM, 400 μL ; prepared as mentioned in the methods section) in an Eppendorf tube. The resulting mixture was gently homogenized by pipetting and transferred into a 1 mL Hellma quartz cuvette. The hydrogel was allowed to solidify at 25°C for 15 minutes, resulting in a final hydrogel concentration of approximately 0.66 wt%.

The enzyme cascade reactions were started by adding 400 μL of Milli-Q water to the hydrogel-containing cuvette, followed by the immediate addition of a 15 μL substrate solution (glucose 100 mM, *o*-PD 50 mM, mixed in a 1:1 volume ratio).

The time-dependent enzyme-mediated cascade synthesis of fluorescent 2,3-DAP was monitored for 60 min by fluorescence spectroscopy ($\lambda_{\text{exc}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 550\text{-}625 \text{ nm}$, 1 spectrum per minute).

2.7.4 Supplementary figures

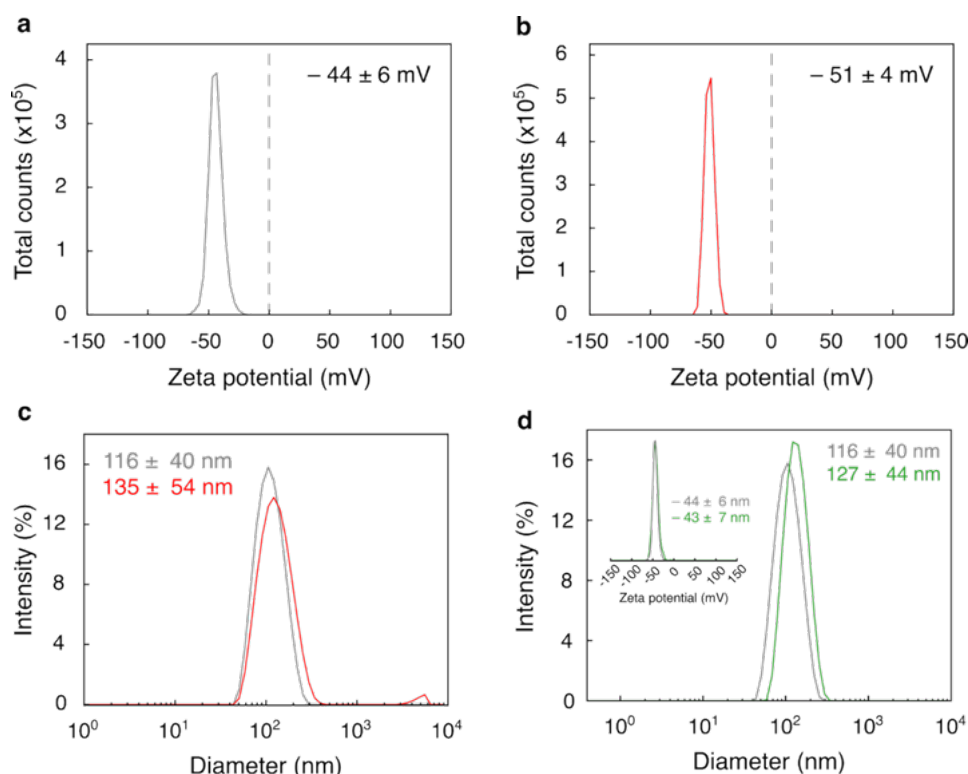


Figure S6 Zeta potential measurement for the 1:4 PDMAEMA/PAA (10 mM) molar ratio mixture prior to shaking. **b**, Zeta potential measurement for the same 1:4 PDMAEMA/PAA (10 mM) mixture after 30 s of manual shaking, indicating an increase in negative surface charge after agitation. **c**, Size distribution profiles of the 1:4 PDMAEMA/PAA (10 mM) mixture before (grey plot) and after (red plot) manual shaking. After shaking, DLS continues to show the presence of nano-coacervates, and a small peak appears in the micrometer size range indicating formation of much larger structures. **d**, Size distribution profiles of 1:4 PDMAEMA/PAA (10 mM) nano-coacervates, freshly prepared (grey plot) and after 7 days left undisturbed (green plot). Nano-coacervates persist with comparable diameters after one week. The inset reports their corresponding normalized zeta potential analysis, indicating no significant changes. These results confirm that nano-coacervates are stable over long time, with their strong negative charge preventing coalescence.

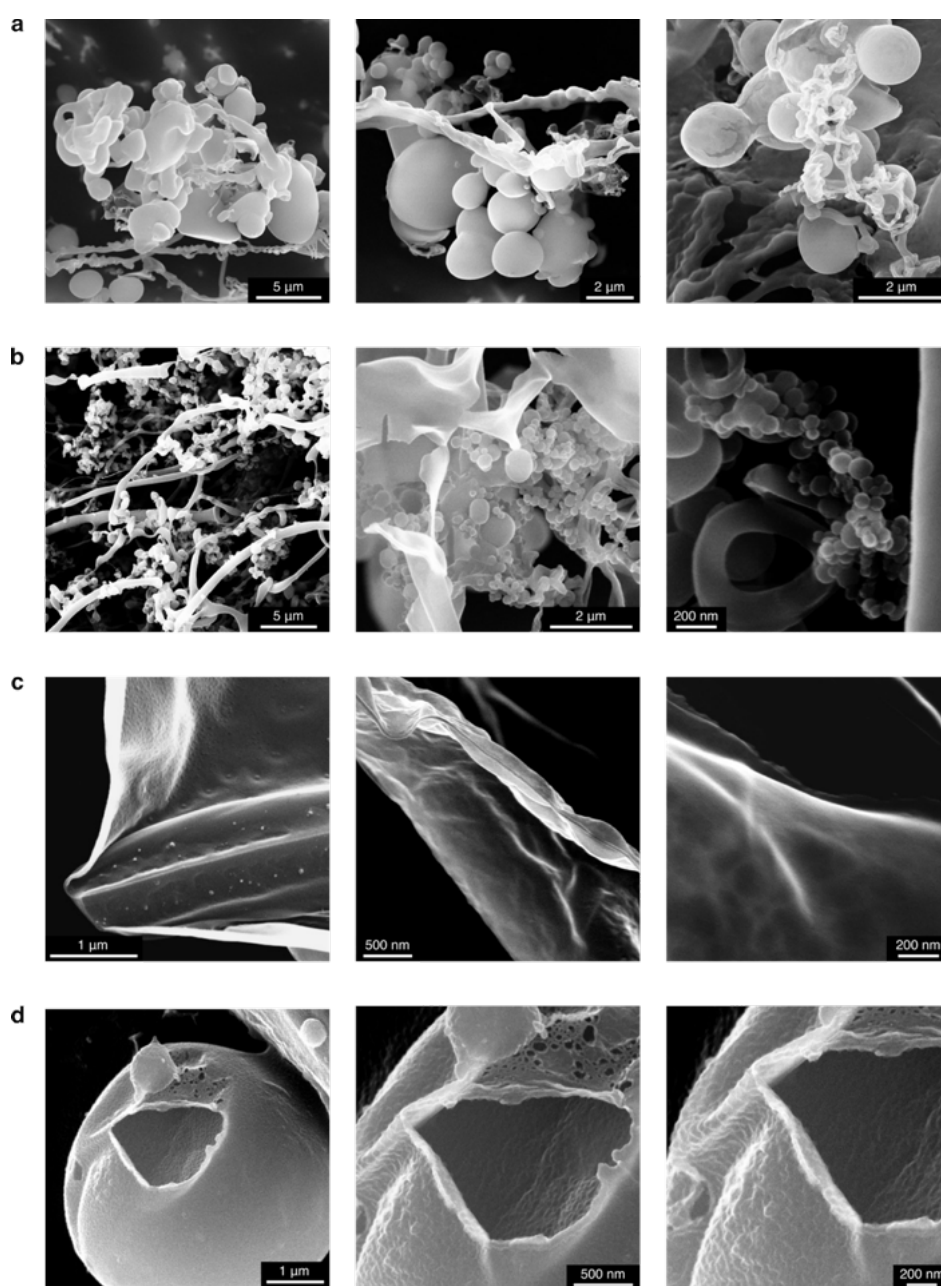


Figure S7 Representative Cryo-SEM images of the PDMAEMA/PAA system at various molar ratios after sample sublimation. **a**, Coacervate microdroplets ($>1\ \mu\text{m}$ in diameter) were observed at a 1:1 PDMAEMA/PAA (10 mM) molar ratio. **b**, Numerous distinct nano-coacervates ($\approx 100\ \text{nm}$ in diameter) are observed were observed at a 1:4 PDMAEMA/PAA (10 mM) molar ratio and before manual shaking. **c**, Membrane details of coacervate vesicles formed from a 1:4 PDMAEMA/PAA (10 mM) molar ratio after 30 s of manual shaking. **d**, Detailed Cryo-SEM images of a single coacervate vesicle.

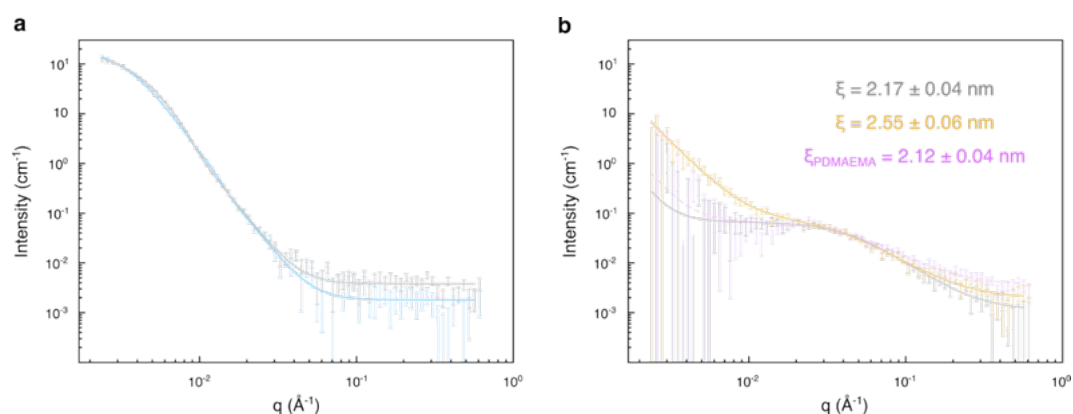


Figure S8 SANS analysis of PDMAEMA/PAA (10 mM) mixtures in D_2O at varying polymer molar ratios. Each graph shows the scattering data before (grey curve and error bars) and after (colored curve and error bars) the application of an impulsive force. **a**, SANS data of nanocoacervates formed at a 1:4 PDMAEMA/PAA (10 mM) molar ratio. **b**, SANS data of coacervate microdroplets formed at a 1:1 PDMAEMA/PAA (10 mM) molar ratio. The correlation length value slightly decreased from $\xi = 2.17 \pm 0.04$ nm to $\xi = 2.55 \pm 0.06$ nm upon shaking. The upward shift in the low- q region post-shaking suggests clustering and the formation of larger coacervate droplets. In general, these results are comparable with those obtained from PDMAEMA semi-diluted solution in D_2O , which is reported in the graph as a pink dashed curve. In this latter case, the correlation length value is $\xi = 2.12 \pm 0.04$ nm.

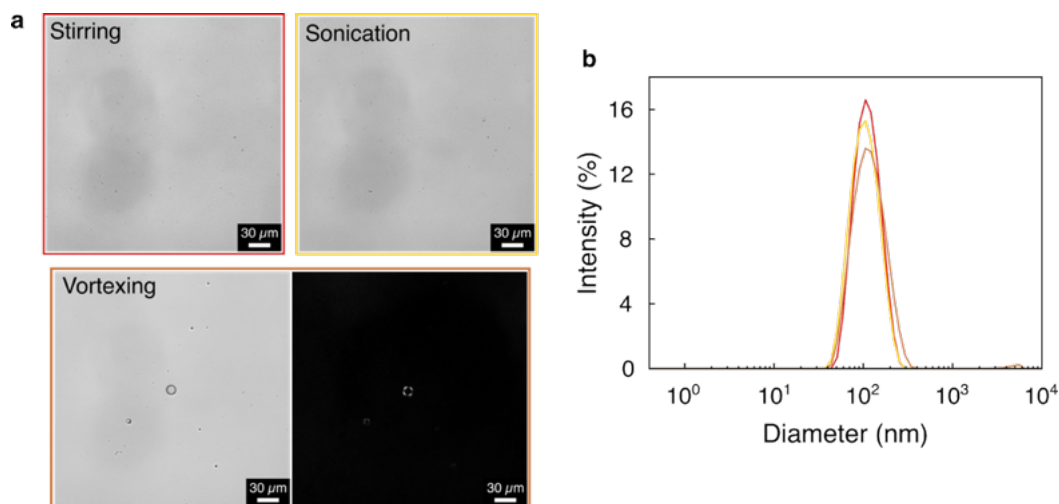


Figure S9 a, Microscopy and **b**, DLS analysis of 1:4 PDMAEMA/PAA (10 mM) molar ratio mixture after being subjected to various forms of mechanical energy for 30 s.

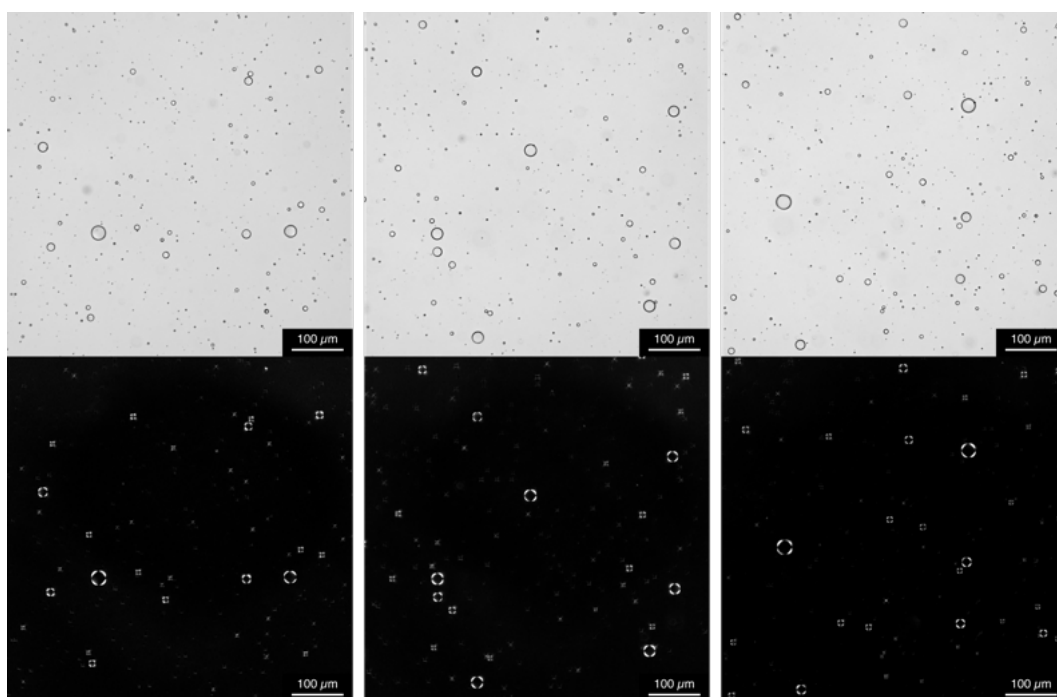


Figure S10 Gallery of representative microscopy images for the size distribution study of a population of a 1:4 PDMAEMA/PAA (10 mM) molar ratio coacervate vesicles. Each microscopy image was analyzed using both high resolution brightfield microscopy and cross-polarized light microscopy.

2 | MECHANICALLY DRIVEN DISSIPATIVE SELF-ASSEMBLY OF COACERVATE VESICLES

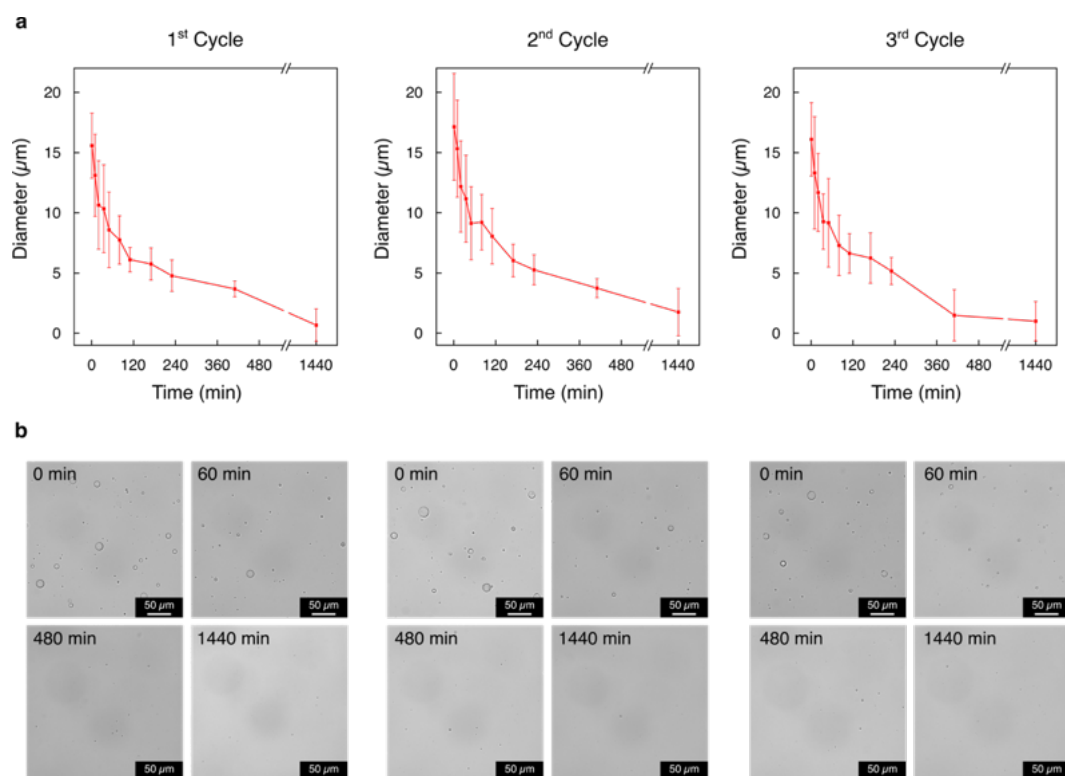


Figure S11 Reversible formation of coacervate vesicles from a 1:4 molar ratio mixture of PDMAEMA/PAA (10 mM). **a**, Progressive decrease in coacervate vesicle diameter over a 24-hour period (1440 minutes). To accelerate the dissolution process, samples were kept under constant agitation using a tube revolver throughout the entire duration of the experiment. Observations began immediately after manual shaking ($t = 0$ min) and continued at regular intervals. The 2nd and 3rd cycles represent the same vesicle system, which was manually shaken again after a period of 24 and 48 hrs, respectively. In each of these cycles, a quasi-exponential decay in vesicle diameter is observed, with vesicle size initially averaging around 15 μm and decreasing gradually to complete disassembly. Error bars = standard deviation ($n=25$). **b**, Representative brightfield microscopy images at selected time points (0, 60, 480, and 1440 minutes) for each of the three cycles.

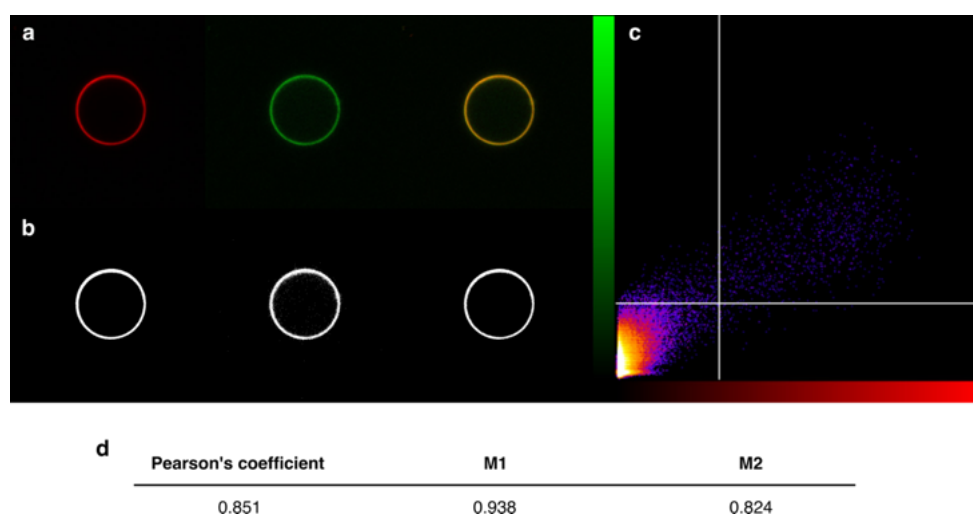


Figure S12 Colocalization studies of coacervate vesicles formed from fluorescent polymers. Coacervate vesicles were prepared at a 1:4 PDMAEMA/PAA (10 mM) molar ratio, each 10 mM polymer stock solution contained a 10 mol% of the corresponding fluorescently tagged polymer, FITC-PDMAEMA or RITC-PAA. **a**, Red, green, and merged channels of a confocal laser scanning microscopy image of a single representative coacervate vesicle. **b**, Binary thresholded images of the red, green, and merged channels showing pixel overlap. **c**, Colocalization scatterplot of the individual pixels from the paired images, with white lines indicating threshold levels for both channels. Most pixels above the threshold values are aligned in the top-right quadrant, suggesting nearly complete colocalization. **d**, Pearson's correlation coefficient and Mander's coefficients (M1, M2) for FITC-PDMAEMA and RITC-PAA colocalization. Both Pearson's and Mander's coefficient values approach 1, indicating that nearly all pixels of the two thresholded channels overlap, demonstrating high colocalization of the two polymers. Colocalization parameters were quantified using the BIOP JACoP plugin in ImageJ, applying an automatic threshold set by the Otsu method.

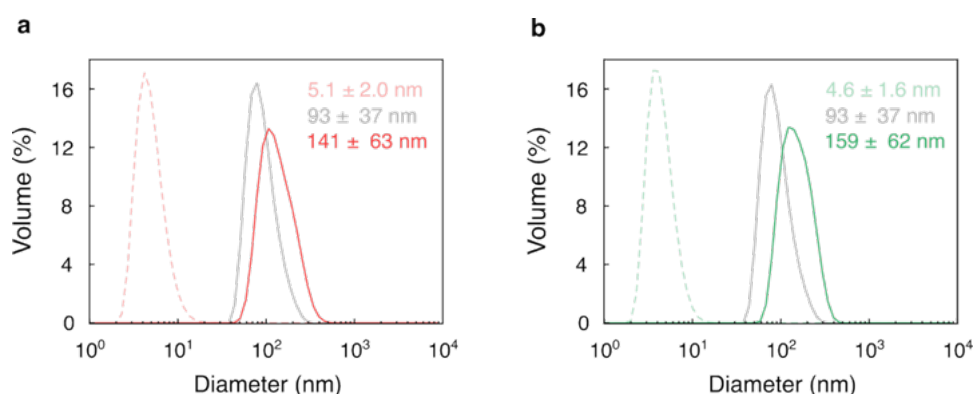


Figure S13 Dynamic light scattering (DLS) analysis of enzyme-loaded nano-coacervates. **a**, Size distribution profiles comparing: PDMAEMA/PAA (1:4 molar ratio) nano-coacervates (gray

2 | MECHANICALLY DRIVEN DISSIPATIVE SELF-ASSEMBLY OF COACERVATE VESICLES

plot), the same coacervate system but with encapsulated GOx (red plot), and a 1 mg mL⁻¹ GOx solution in H₂O at pH 6.5 (dashed light red plot). **b**, Size distribution profiles comparing: PDMAEMA/PAA nanodroplets (gray plot), the same nanodroplets but containing HRP (green plot), and a 1 mg mL⁻¹ HRP solution in H₂O at pH 6.5 (dashed light plot green).

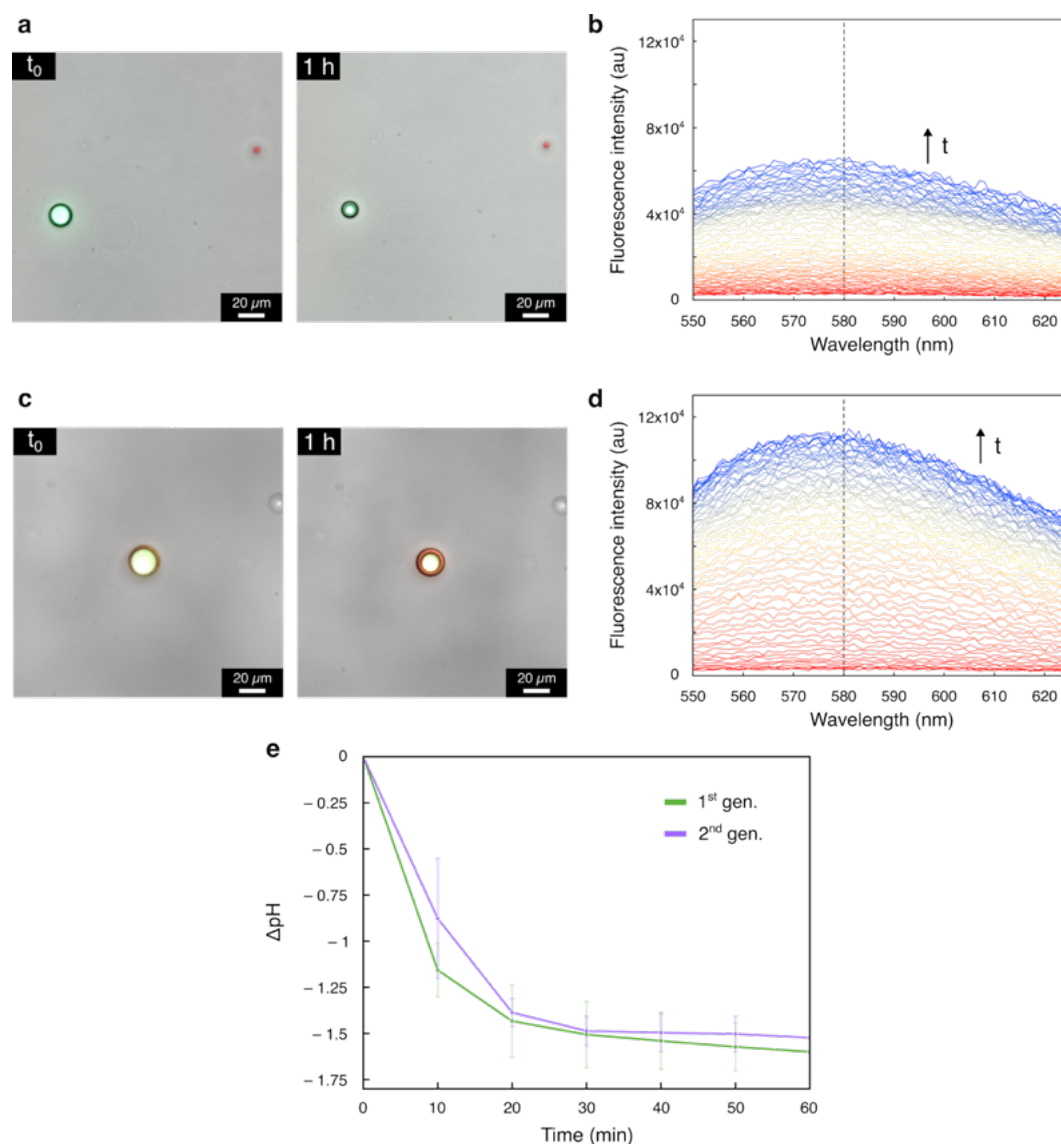


Figure S14 Hydrogel-embedded enzyme-loaded coacervate vesicles and cascade reaction kinetics. **a**, Representative optical microscopy images with overlaid brightfield, red and green fluorescence channels showing a mixed binary population of coacervate vesicles containing only RITC-tagged GOx or FITC-tagged HRP (1st generation) and embedded in an agarose hydrogel matrix. The image on the left was captured immediately after vesicle formation, the image on the right was acquired on the same position after 1 hr. The image after 1 hr shows that within the hydrogel the vesicles behave like in the bulk solution, with their diameter gradually decreasing overtime and without detectable enzyme leakage.

b, Kinetic emission spectra of the 1st generation coacervate vesicles following the addition of substrates (glucose and o-PD). The formation of 2,3-DAP was monitored by tracking the emission peak at 580 nm, providing insights into the cascade reaction rate under segregated conditions. **c**, Optical microscopy images (brightfield, red and green channels overlapped) of coacervate vesicles containing both RITC-tagged GOx and FITC-tagged HRP (2nd generation) embedded within an agarose hydrogel matrix. The image on the left was captured immediately after vesicle formation, the image on the right was acquired on the same position after 1 hr. The enzymes display selective localization, with GOx predominantly at the vesicle membrane and HRP within the internal lumen, similarly to what observed in aqueous conditions. **d**, Kinetic emission spectra for the 2nd generation coacervate vesicles, showing faster 2,3-DAP production due to enzyme co-encapsulation within the same vesicle, which facilitates faster reaction kinetics due to the reduced diffusion distance between catalytic sites. Experiments were performed in triplicate, with results summarized in **Fig. 5f**. **e**, Time-dependent pH variations during enzymatic cascade reaction in 1st generation (green plot) and 2nd generation (purple plot) coacervate vesicles. The decrease in pH is associated with the production of gluconic acid following the hydrolysis of gluconolactone. The comparable kinetics between the two systems confirms similar GOx activity, demonstrating that the enhanced overall reaction rate of 2,3-DAP production in the 2nd-generation system arises specifically from improved H₂O₂ diffusion rather than differences in the first catalytic step. Error bands represent standard deviation ($n = 3$).

3

STIMULI-RESPONSIVE COACERVATE DROPLETS INTEGRATING PHOTOELECTRON TRANSFER MACHINERIES

The work presented in this chapter was conducted under the supervision of Prof.
P. Gobbo.

3.1 INTRODUCTION

Enzymatic processes are indispensable for sustaining life, as the maintenance of biological function requires thousands of chemical transformations to proceed with high rates and remarkable selectivity. Living systems continuously sense, process, and classify information from their environment through complex enzymatic reaction networks.^{1,2} These networks respond to a wide range of physical and chemical stimuli, including nutrient availability, pH variations, temperature changes, and fluctuations in light intensity.³ While enzymatic regulation in cellular systems is inherently complex, such behavior can often be rationalized in terms of individual enzymatic reactions that communicate with one another within interconnected networks.^{4,5} Ultimately, the kinetics of each elementary enzymatic step determines the overall system response, both in terms of outcome and temporal evolution.

Enzymes are highly sensitive to their chemical environment and operate within well-defined activity windows. Owing to their pronounced amphoteric character, arising from the presence of numerous acidic and basic functional groups, one of the most critical parameters influencing enzymatic activity is pH.

3.1.1 Urea-urease

The rate of an enzymatic reaction typically follows a bell-shaped dependence on pH, with a maximum corresponding to optimal catalytic activity. pH-responsive enzymatic systems can display complex dynamics due to the intrinsic nonlinearity of their kinetics.⁶ A well-established and extensively studied example of such behavior is provided by the urea-urease reaction network (**Figure 3.1a**). Urease catalyzes the hydrolysis of urea into carbon dioxide and ammonia, leading to an increase in pH, and exhibits maximal activity around pH 7 (**Figure 3.1b**).⁷ When the reaction is initiated under acidic conditions, the enzyme progressively basifies the solution, thereby enhancing its own activity through positive feedback until reaching pH values around 7. As the reaction proceeds further and the pH continues to rise, enzymatic activity

decreases again, and the system ultimately stabilizes at a final pH of approximately 8.5, corresponding to the ammonia/ammonium buffering regime. This autocatalytic followed by self-inhibitory behavior produces a characteristic sigmoidal pH-time profile, in which the sharp transition between low- and high-pH states is commonly referred to as the “clock time” (**Figure 3.1c**).^{8–10}

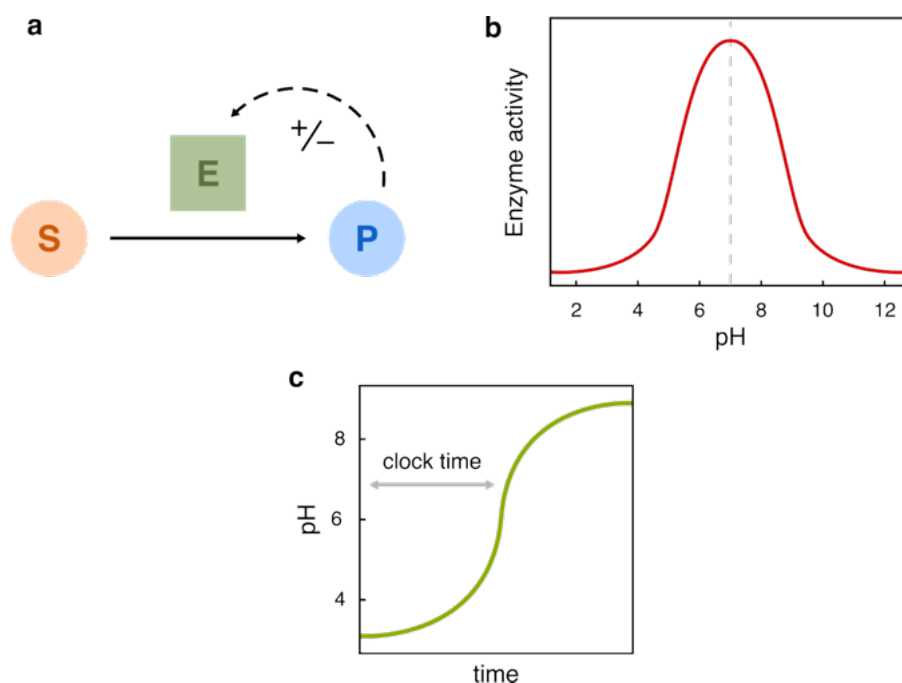


Figure 3.1 a, Schematic representation of the autocatalytic and self-deactivating urea-urease reaction network. The substrate S (urea) is converted into the product P (ammonia) by the enzyme E. At low pH, P acts as an activator of E, increasing enzymatic activity. Above pH 7, P progressively acts as a deactivating agent. **b**, Bell-shaped pH dependence of urease activity, with a maximum around pH 7. **c**, Autocatalytic sigmoidal pH versus time profile characteristic of the urea-urease reaction network.

The urea-urease network has been widely coupled with supramolecular systems to drive pH-mediated processes in a nonlinear and time-programmed manner.¹¹ Che *et al.* developed urease-containing microgels and polymersomes that undergo reversible swelling and contraction in response to transient pH changes, generating self-regulated, out-of-equilibrium feedback loops.^{12,13} In combination with gelling systems, urease has also been employed to program sol-gel transitions and to create stimuli-responsive materials.^{14–16} More recently, urease-driven pH changes have been used to

induce non-Newtonian behavior in polyelectrolyte systems, such as shear thickening at low pH and shear thinning at high pH.¹⁷

Under appropriate conditions, these systems can even exhibit oscillatory behavior when urea is combined with an acidic co-fuel. Notably, theoretical studies have predicted that truly autonomous oscillations may arise when the urea-urease network is confined within open reactors that allow differential transport of urea and protons.^{18,19} While such spontaneous oscillatory dynamics have been demonstrated theoretically, they have not yet been experimentally realized and remain an open challenge in the field.²⁰

3.1.2 Photoinduced electron transfer supramolecular sensors

Photoinduced electron transfer (PET) is a fundamental photophysical process that involves the formation of a charge-separated state consisting of a donor radical cation and an acceptor radical anion.^{21–23} PET can also happen intramolecularly when donor and acceptor coexist in the same molecule.²⁴ These molecules can be designed to present PET-based fluorescent behaviors.

A typical PET-based fluorescent molecule consists of three key elements: a fluorophore, an appropriate linker, and a receptor (Figure 3.2). In most cases, electron transfer occurs from the receptor to the fluorophore, as the highest occupied molecular orbital (HOMO) of the receptor lies energetically between the HOMO and the lowest unoccupied molecular orbital (LUMO) of the fluorophore. Upon photoexcitation, an electron can be transferred from the receptor to the excited fluorophore, generating a charge-separated state. In this configuration, relaxation of the excited fluorophore to the ground state via fluorescence is inhibited, resulting in emission quenching. Interaction between a guest species and the receptor lowers the energy of the receptor HOMO below that of the fluorophore HOMO, thereby

suppressing the electron transfer process. When PET is suppressed, fluorescence emission is restored, providing a sensitive readout mechanism.

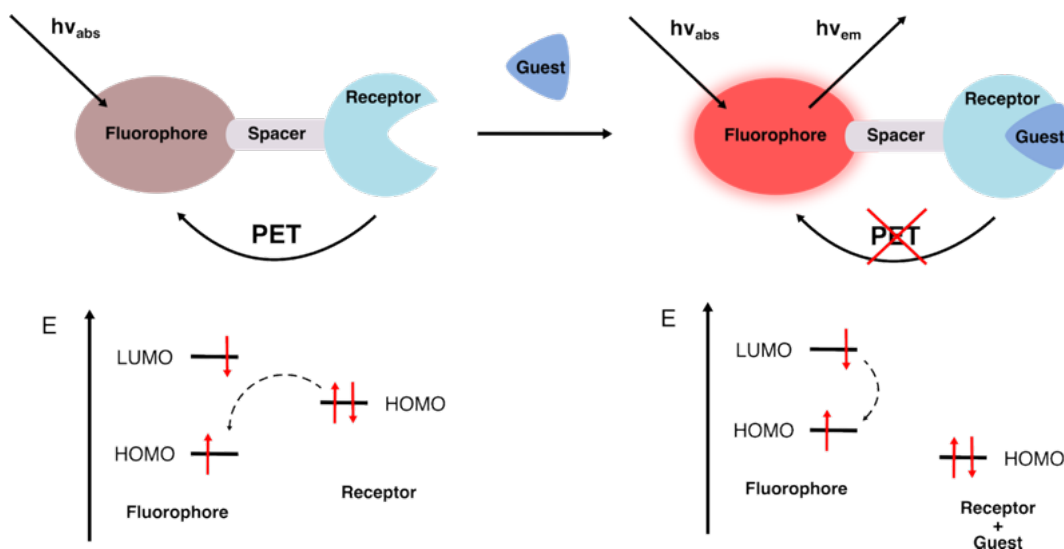


Figure 3.2 Schematic representation of a PET-based fluorescent molecular system. Interaction with a guest species suppresses the PET process, resulting in fluorescence activation of the fluorophore. The lower panel shows the corresponding modification of the frontier molecular orbital energy levels underlying the PET mechanism.

This principle has been widely exploited in the design of molecular fluorescence sensors and probes.²⁵ The first PET-based sensor was reported by de Silva in 1985 and consisted of aminomethyl anthracene derivatives responsive to pH variations.²⁶ Since then, the field has expanded significantly, encompassing a broad range of molecular architectures and target analytes. PET sensors have been extensively employed for the detection and imaging of intracellular processes in living systems,²⁷ including the sensing of biologically relevant metal ions such as Cd^{2+} , Zn^{2+} , Hg^{2+} , Cu^{2+} , Ag^{+} .²⁸ They have also been developed to monitor reactive oxygen species, which are closely associated with pathological conditions,²⁹ as well as intracellular pH variations that strongly influence cellular functionality, growth, and division.³⁰ In many of these systems, amine-based receptor units serve as proton-binding sites, allowing PET modulation through reversible protonation.³¹

Because PET sensors are constructed using modular synthetic strategies, their molecular structure and functionality can be readily tailored. This synthetic flexibility enables the systematic exploration of new architectures, signal transduction mechanisms, and sensing capabilities, making PET-based systems particularly attractive for integration into complex, responsive chemical networks.

3.1.3 Coacervate droplets as enzymatic microreactors

LLPS can markedly enhance reaction rates by creating crowded microenvironments that modulate diffusion and local concentrations.^{32,33} This property is particularly advantageous for enzymatic reactions. Coacervates can accelerate enzyme-catalyzed processes by co-sequestering enzymes and substrates within a dense phase, thereby increasing effective concentrations and reducing diffusion distances.^{34,35}

Urease has been successfully incorporated into several coacervate-based systems. Yin *et al.* reported alginate/silk fibroin coacervate microdroplets capable of reversibly forming vesicles in response to pH changes.³⁶ In that work, sequestration of urease and glucose oxidase enabled substrate-triggered droplet-to-vesicle transitions upon addition of urea or glucose. In a different approach, Rowland *et al.* employed urease within a hierarchical double-coacervate system composed of PAA/polyaspartate and dextran/PEG to drive calcium carbonate mineralization.³⁷ There, enzymatically generated carbonate ions induced CaCO₃ microsphere formation, highlighting how coacervate properties influence biomineralization pathways. Despite these advances, the intrinsic pH-dependent activity of urease and its nonlinear feedback on the surrounding coacervate environment has not yet been systematically explored. In particular, how enzyme-driven pH changes can dynamically alter coacervate properties and be harnessed to generate functional responses remains an open question.

In this chapter, we address this gap by introducing a new class of coacervate microdroplets incorporating photoinduced electron transfer polymers. These droplets respond sensitively to pH variations and enable the urea-urease reaction network to be transduced into a visible fluorescence signal. By coupling enzymatic activity to optical readout within a confined, phase-separated environment, this system establishes coacervate microdroplets as active enzymatic microreactors capable of converting chemical dynamics into accessible information, thereby setting the stage for the experimental investigations that follow.

3.2 PET POLYELECTROLYTES CAN FORM COACERVATE DROPLETS

To investigate how enzymatic reaction networks can be coupled to signal transduction within phase-separated compartments, PET-polymer-based coacervate microdroplets were prepared and systematically characterized. The PET-active monomer piperDMA was synthesized from perylenetetracarboxylic dianhydride, aminoethylpiperazine, and methacryloyl chloride. PiperDMA exhibits a receptor-chromophore-receptor architecture, in which the piperazine moieties act as proton-responsive receptors. Protonation of these receptors blocks the PET process, resulting in strong fluorescence emission (ON state), whereas deprotonation at higher pH values restores PET activity, leading to fluorescence quenching and the formation of large aggregates (OFF state, **Figure 3.3**, Figure S17).

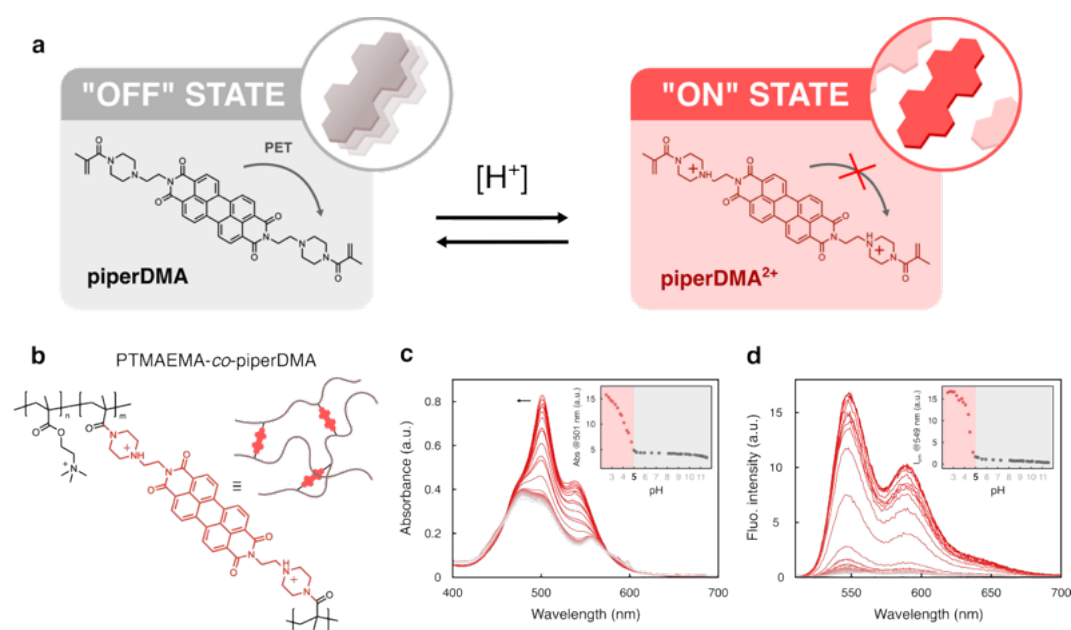


Figure 3.3 a, pH-dependent OFF-ON switch of piperDMA monomer. Protonation of the piperazine units blocks the PET mechanism, allowing fluorescence emission of the perylenic unit. **b**, Structure and schematic representation of PTMAEMA-co-piperDMA. **c**, UV-vis spectra of PTMAEMA-co-piperDMA changing pH. A lowering of the peak at 501 nm with consequent blue shift is observed when increasing the pH. **d**, Fluorescence emission spectra of PTMAEMA-co-piperDMA changing pH. A total quench is observed at pH > 5.

This ON-OFF behavior is preserved upon incorporation of the PET-active monomer into a polyelectrolyte. The copolymer poly(2-(trimethylammoniumethyl methacrylate) chloride) (PTMAEMA)-*co*-piperDMA was synthesized in water and characterized, displaying a clear fluorescence switching transition around pH 5 (Figure 3.3c,d). Analysis of the fluorescence response using a Henderson-Hasselbalch model yielded an apparent pK_a value of 4.4 (Figure S18). Importantly, because the PTMAEMA backbone itself is pH-independent, the observed fluorescence changes can be directly attributed to protonation of the piperDMA moieties.

Coacervate microdroplets were formed by mixing aqueous solutions of PTMAEMA-*co*-piperDMA and ATP at a 1:1 molar ratio (10 mM, pH 3). As commonly observed for non-stabilized coacervate systems, these droplets gradually coalesced, eventually forming a single dense phase at the bottom of the container. As previously

demonstrated by Agrawal *et al.*,^{38,39} the polyelectrolyte-rich supernatant was carefully removed and replaced with ultrapure Milli-Q water. Upon application of mechanical energy via vortexing, this procedure yielded spherical and stable coacervate droplets with diameters ranging from 2 to 30 μm . (Figure 3.4). In contrast to conventional coacervate microdroplets, these reconstituted microcompartments remained stable over extended periods, exhibiting minimal coalescence and negligible wetting of the glass surface. This enhanced stability arises from the removal of excess charged species and counterions from the continuous phase, which promotes the formation of stronger interfacial salt bridges between the oppositely charged components at the coacervate-water interface.⁴⁰ Consistent with this interpretation, replacing ATP with a pH-independent polyanion such as polystyrene sulfonate resulted in disordered aggregates rather than well-defined droplets, due to excessively strong electrostatic interactions between the two species in the initial coacervate phase.⁴¹

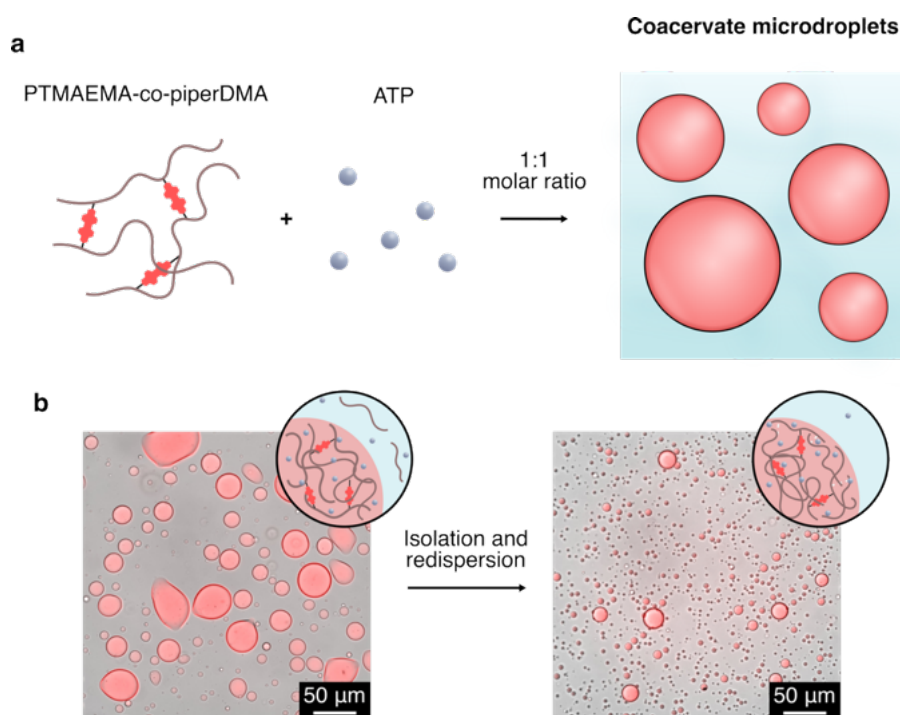


Figure 3.4 **a**, Schematic representation of PTMAEMA-*co*-piperDMA/ATP coacervate droplet formation. **b**, Stabilization of PTMAEMA-*co*-piperDMA/ATP coacervate droplets. Left: merged brightfield and fluorescence microscopy images of a metastable dispersion prior to stabilization, showing progressive droplet coalescence and growth. Right: merged brightfield and fluorescence

microscopy images of a stable droplet population obtained after removal of the supernatant phase and redispersion in ultrapure water.

Strikingly, the stabilized coacervate droplets retained their structural integrity in dilute buffer solutions and remained stable over a broad pH range. This stability enabled investigation of whether the PET functionality of the polycationic component was preserved within the phase-separated environment. Indeed, the droplets exhibited a reversible fluorescence ON-OFF transition around pH 5, mirroring the behavior observed in solution and demonstrating effective transduction of proton concentration into an optical signal within the coacervate microenvironment (**Figure 3.5**).

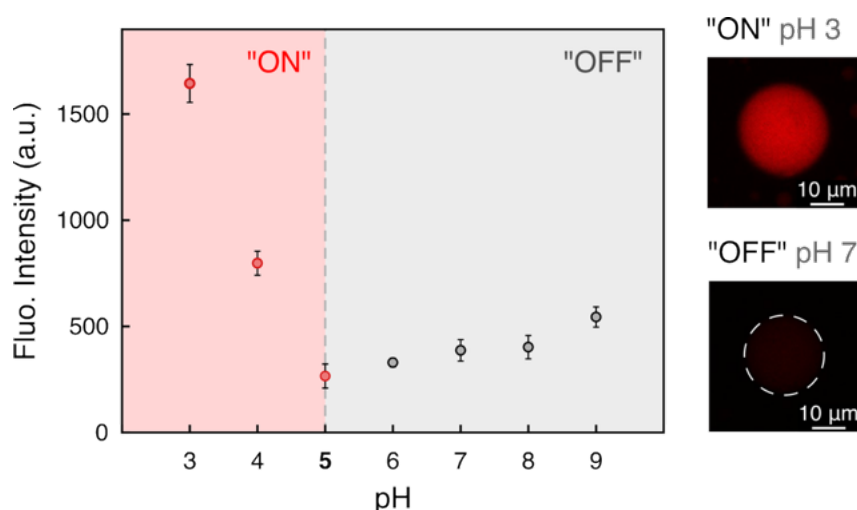


Figure 3.5 Fluorescence intensity profiles extracted from confocal microscopy images of PTMAEMA-*co*-piperDMA/ATP coacervate droplets acquired in different buffer solutions at varying pH values. For pH < 5, the droplets exhibit strong fluorescence, while increasing pH leads to pronounced fluorescence quenching. Representative fluorescence microscopy images at pH 3 and pH 7 are shown on the right. Error bands represent $\pm$ s.d. (n = 5).

3.3 PIPERDMA DROPLETS AS ENZYMATIC MICROREACTORS

With the goal of creating a synthetic protocell capable of sensing internal chemical stimuli and transducing them into optical signals, PTMAEMA-co-piperDMA/ATP coacervate droplets containing urease were prepared. Upon droplet formation, the enzyme was selectively sequestered within the coacervate phase and homogeneously distributed throughout the interior. This behavior was confirmed using FITC-labelled urease, which showed complete colocalization with the fluorescent PTMAEMA-co-piperDMA signal (**Figure 3.6**). Quantitative image analysis yielded a Pearson's correlation coefficient of 0.915 (Figure S20), confirming efficient enzyme encapsulation without phase segregation.

To assess whether confinement within the stabilized droplets compromised molecular mobility, fluorescence recovery after photobleaching (FRAP) experiments were performed. After redispersion in ultrapure water, both PTMAEMA-co-piperDMA and urease retained high internal mobility, as evidenced by rapid fluorescence recovery within less than 60 s following photobleaching. This result indicates that, despite their enhanced structural stability, the droplets remain dynamically liquid-like, a prerequisite for sustained enzymatic activity and efficient mass transport.

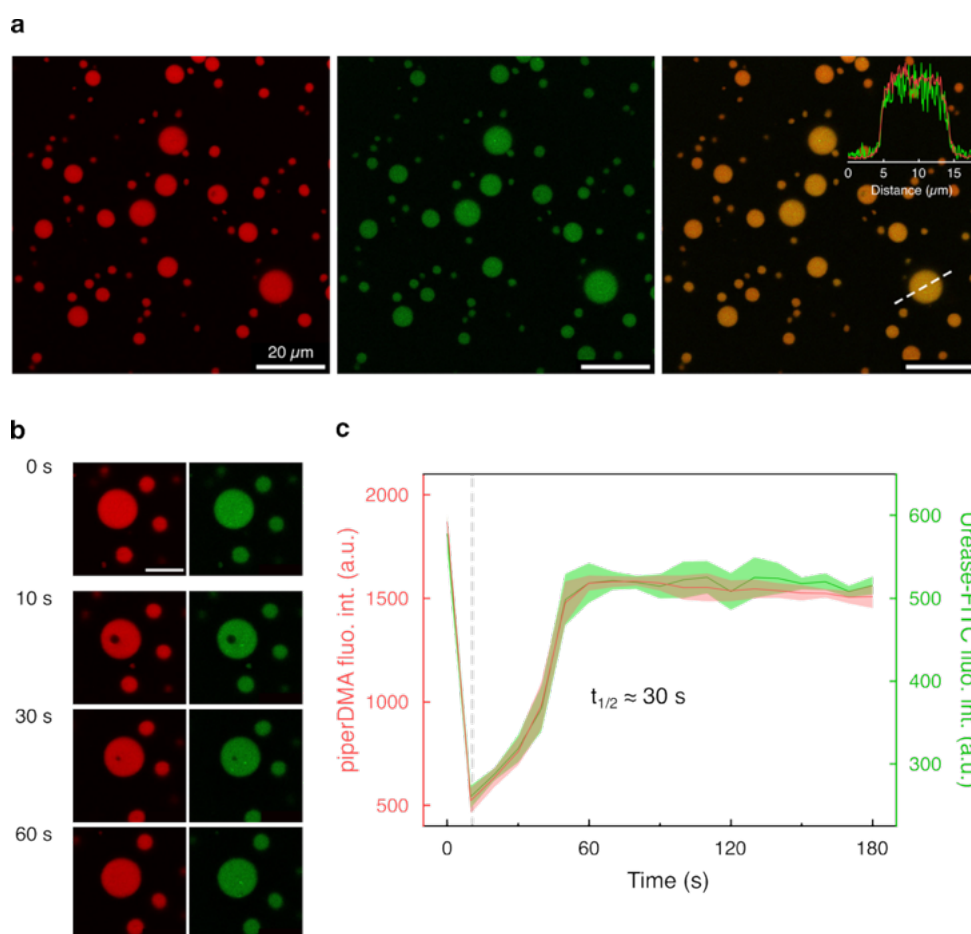


Figure 3.6 a, Confocal fluorescence microscopy images of PTMAEMA-co-piperDMA/ATP coacervate droplets containing FITC-labelled urease. The first image shows the red fluorescence of piperDMA, the second the green emission of FITC-urease, and the third the merged image, demonstrating colocalization within the droplets. The normalized fluorescence intensity line profile shown in the merged image was extracted along the dashed line and highlights the spatial overlap of the two channels. **b**, FRAP analysis of FITC-urease-loaded coacervate droplets, shown at $t = 0$ s (pre-bleaching), 10 s, 30 s, and 60 s. Scale bars: 10 μm. **c**, Normalized fluorescence recovery curves corresponding to b. Both piperDMA and FITC fluorescence were recovered in less than 60 seconds after photobleaching, with a $t_{1/2}$ of 30 s ca. Error bands represent $\pm$ s.d. (n = 3).

Building on the efficient sequestration and mobility of urease within the droplets, we next explored the catalytic activity of the enzyme upon addition of urea. The urea-urease reaction provides a well-defined, pH-responsive network, enabling us to directly probe how enzymatic work performed inside the droplets translates into local pH variations and, ultimately, into a measurable fluorescent output (Figure 3.7a).

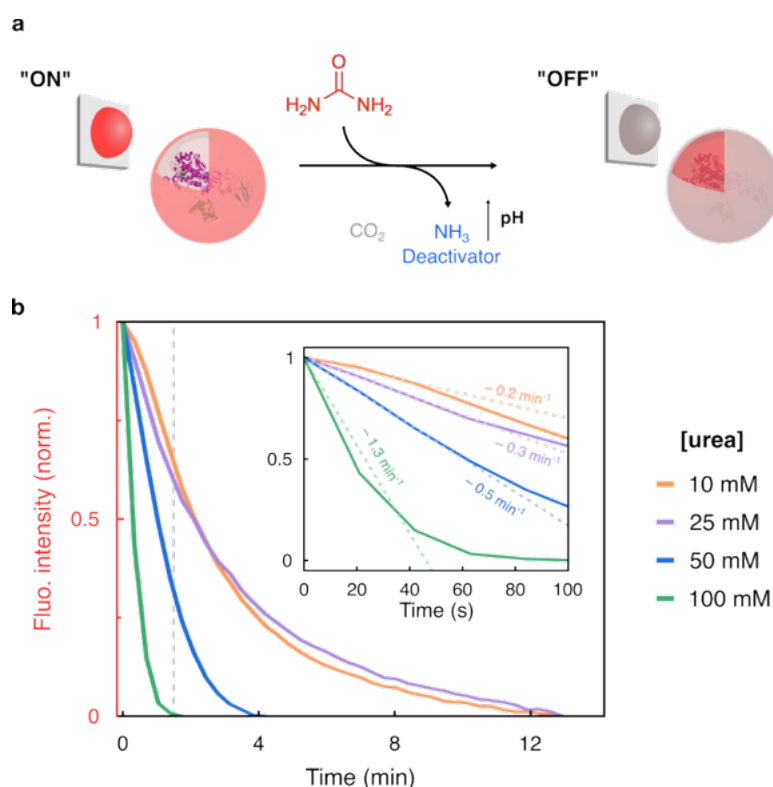


Figure 3.7 a, Schematic representation of the urea-urease reaction network inside the coacervate droplets in water. From the starting ON state, the continuous production of ammonia from urea increases the pH, finally leading to the OFF state with consequent fluorescence quench. **b**, Fluorescence over time kinetics for PTMAEMA-*co*-piperDMA/ATP coacervate droplets containing urease at varying starting concentrations of urea. In the inset, the same graph is shown for the first 100 seconds of reactions with the corresponding initial rate for each case.

PET-driven fluorescence transduction was first monitored for the system in ultrapure water. Upon addition of urea, the urease sequestered within the droplets started producing ammonia, inducing a progressive increase in pH (Figure 3.7b). By varying the initial urea concentration from 10 to 100 mM (corresponding to final concentrations of 3–30 mM in solution), distinct kinetic behaviors were observed. In all cases, the fluorescence intensity of the droplets decreased with slopes and characteristic times that depended on the urea concentration. The time required for fluorescence quenching decreased from approximately 12 min at 10 mM urea to about 1 min at 100 mM urea. Analysis of the initial rates of fluorescence change revealed a clear dependence on substrate concentration. The observed fluorescence quenching

rates scale with urea concentration in a manner consistent with Michaelis-Menten kinetics, with deviations at higher substrate concentrations reflecting the coupled nature of enzymatic activity, and pH-sensitive fluorescence readout., yielding a value of $K_M = 15$ mM (7.5 mM not considering the higher concentration of urea, Figure S21), in agreement with previously reported values for urease in aqueous environments.⁴² These preliminary results demonstrate that PET-based coacervate droplets enable quantitative, real-time monitoring of enzymatic reaction networks and that fluorescence microscopy can provide reliable access to kinetic parameters of enzymes operating within phase-separated protocellular compartments. Further studies in this direction are currently ongoing to establish a quantitative correlation between coacervate dynamics and the kinetics of the embedded enzymatic reaction.

3.4 OSCILLATORY ENZYMATICAL REACTIONS

Building on the ability of PET-based coacervate droplets to transduce enzymatic activity into a fluorescent signal, we next explored the possibility of developing an autonomous system capable of sustained or transient fluorescence oscillations in response to enzymatic reaction networks. Such behavior would represent a significant step toward life-like protocells, as oscillations emerge from the interplay between non-linear reaction kinetics, transport processes, and compartmentalization under non-equilibrium conditions. The urea-urease reaction network is a particularly attractive candidate in this context. When coupled to appropriate buffering conditions and mass transport, this system has been theoretically predicted to support spontaneous pH oscillations.⁹ These dynamics are expected to be stochastic in nature and strongly dependent on the availability and differential transport of fuels (urea and protons) within an open reactor.¹⁸

Based on these considerations, we conceptualized an oscillatory platform in which enzyme-loaded PET-based coacervate droplets act simultaneously as microreactors and signal-transduction units, enabling real-time visualization of reaction dynamics. The droplets were found to be stable in low-concentration acidic buffer (pH 3, Figure

3.5), which serves both as an external proton reservoir and as a means to maintain the PET system in its fluorescent ON state. Upon addition of urea, urease activity inside the droplets produces ammonia, locally increasing the pH and switching the PET polymer to the OFF state, thereby quenching fluorescence. Subsequent proton diffusion from the surrounding buffer can, in principle, restore acidic conditions, reactivate the enzyme, and close the cycle, potentially leading to oscillatory behavior (Figure 3.8).

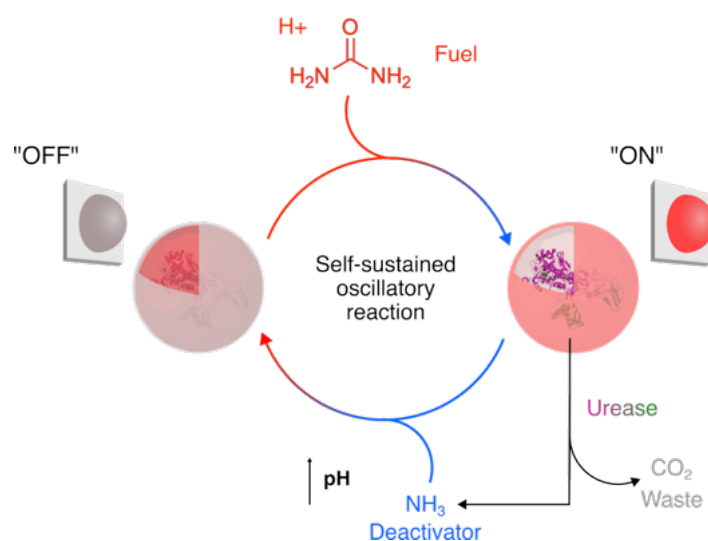


Figure 3.8 Schematic representation of the self-sustained oscillatory system involving PET-based droplets. Protons present in the acidic buffer and urea act as fuel components: the former activates the PET-based coacervate droplets, while the latter serves as substrate for the enzyme. Enzymatic conversion of urea into ammonia increases the local pH, leading to droplet deactivation and closing the feedback cycle.

As a control, this concept was first examined in ultrapure water, where no buffering capacity is present. In agreement with the expected behavior and with the results discussed in Figure 3.7, the system exhibited a single fluorescence quenching event followed by a stable plateau, with no oscillations observed over a 30 min time window (Figure 3.9a). This behavior is consistent with irreversible enzyme deactivation at elevated pH in the absence of proton replenishment. In contrast, preliminary experiments conducted in 5 mM citrate buffer at pH 3 revealed more complex dynamics. After an initial rapid decrease in fluorescence associated with ammonia

production, fluctuations in fluorescence intensity were observed at longer timescales (Figure 3.9b). These fluctuations are tentatively attributed to proton diffusion from the buffer into the droplets, leading to partial reactivation of urease and renewed enzymatic activity. While these observations do not yet constitute fully developed or sustained oscillations, they provide experimental indications that coupling buffering, transport, and compartmentalization within PET-based coacervate droplets may enable oscillatory enzymatic behavior.

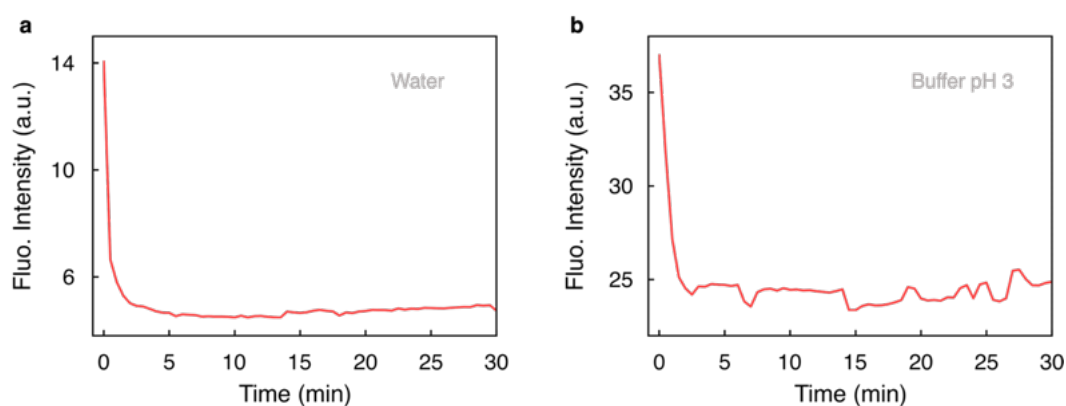


Figure 3.9 Fluorescence intensity over time for the urea-urease network inside piperDMA droplets in (a) ultrapure water and (b) in citrate buffer pH 3.

Overall, these results establish a conceptual and experimental framework for the development of autonomous oscillatory protocells and highlight the key parameters that must be controlled to achieve robust dynamics. Ongoing work is focused on systematically tuning buffer strength and fuel supply to better understand and stabilize these oscillatory regimes.

3.5 CONCLUSIONS AND PERSPECTIVES

In this chapter, PET-based coacervate microdroplets were developed as active enzymatic microreactors capable of coupling chemical reaction networks to optical signal transduction. By assembling complex coacervate droplets from a pH-responsive photoinduced electron transfer polyelectrolyte, we established a system in which

internal biochemical activity can be directly translated into visible fluorescence changes. The PTMAEMA-co-piperDMA/ATP binary polyelectrolyte mixture was shown to form stable coacervate droplets upon mechanical stabilization, preserving both structural integrity and molecular mobility over a broad pH range. Importantly, the PET functionality of the polymer was retained within the dense coacervate phase, allowing the droplets to reversibly switch between fluorescent ON and OFF states in response to protonation.

Urease was efficiently sequestered within the droplets and remained catalytically active and dynamically mobile, as demonstrated by fluorescence colocalization and FRAP experiments. Upon addition of urea, the urea-urease reaction network generated local pH changes that were successfully transduced into a time-dependent fluorescence response. The sigmoidal fluorescence quenching profiles observed at different urea concentrations reflect the intrinsic non-linear kinetics of the enzymatic network, and kinetic parameters extracted from fluorescence data were consistent with literature values. These results demonstrate that PET-based coacervate droplets can function not only as confined reaction vessels but also as self-reporting protocellular systems.

Finally, preliminary experiments explored the integration of buffering and mass transport to move toward autonomous behavior, revealing transient fluorescence oscillations under acidic buffer conditions. While still under development, these results provide an important proof-of-concept that enzymatic reaction networks embedded within coacervate droplets can exhibit emergent, non-equilibrium dynamics when coupled to appropriate environmental reservoirs.

Overall, this chapter establishes PET-functionalized coacervate droplets as a versatile model for constructing synthetic protocells that combine compartmentalization, enzymatic activity, and information transduction. By linking chemical work to optical readout within a soft, dynamic material, this system lays the groundwork for future studies on autonomous behavior, feedback regulation, and chemically driven information processing in protocellular matter.

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3.7 APPENDICES

3.7.1 Materials and methods

Milli-Q water was obtained from a Millipore Milli-Q Plus 185 apparatus and presented a resistivity of 18.2 MΩcm. All solvents were purchased in analytical grade from Sigma-Aldrich and used without further purification. All reagents were used as received unless otherwise stated. Adenosine 5'-triphosphate disodium salt hydrate (ATP), urease (lyophilized 5 U/mg), urea, 3,4,9,10-perylenetetracarboxylic dianhydride, 1-(2-aminoethyl)piperazine, methacryloyl chloride, [2-(methacryloyloxy)ethyl]trimethylammonium chloride solution, triethylamine, and 4,4'-Azobis(4-cyanovaleric acid) (ACVA) were purchased from Sigma-Aldrich.

^1H and ^{13}C NMR spectra were recorded on a Jeol GX-400 MHz spectrometer. ^1H NMR spectra are reported as δ in units of parts per million (ppm) relative to TFA (δ 11.50 s). The number of protons (n) for a given resonance is indicated as nH and was based on spectral integration values. ^{13}C NMR spectra are reported as δ in units of parts per million (ppm) relative to TFA-d (δ 164.29).

Optical and fluorescence microscopy was performed on an Axio Observer 7 (Zeiss) microscope at 20 $\times$ and 40 $\times$ magnification. The microscope was equipped with an Orca Flash 4.0 V3 CMOS camera (Hamamatsu) and a motorized XYZ sample stage. The brightfield images were obtained using a transmitted light LED lamp. Images were analyzed using the latest version of ImageJ software.

Confocal laser scanning microscopy images were obtained on a FV3000 (Olympus) confocal laser scanning microscope. The system was equipped with 4 excitation laser lines: 405, 488, 561, and 640 nm (Coherent), a galvanometric scanning head, and 4 spectral detectors, allowing for simultaneous imaging of up to 4 fluorescence channels. Brightfield images were acquired using a scanning laser line and a transmitted light detector. The inverted microscope body (Olympus IX 83) included a motorized

automated sample stage for multi-area tiled imaging. The following objectives were used: UPLXAPO 20×/0.8 NA (Olympus), and an oil objective UPLXAPO 60×/1.42 NA (Olympus). Images were taken using excitation wavelengths of 488 nm (FITC), and 561 nm (RITC) and emission wavelengths of 500-550 nm, and 575-650 nm, respectively. Images were analyzed using the latest version of ImageJ software.

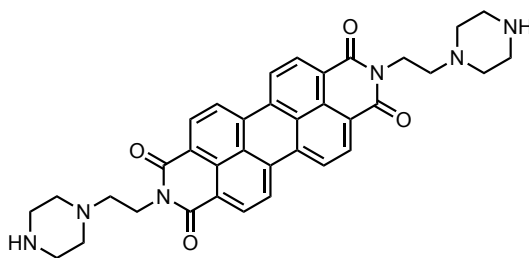
UV-vis spectroscopy was conducted using an Agilent Cary 3500 UV-Vis spectrophotometer, in the wavelength range of 200–800 nm. The instrument was equipped with tungsten-halogen lamps for the visible range and deuterium arc lamps for the UV range.

Fluorescence spectroscopy measurements were performed on an Edinburgh Instruments FS5 spectrofluorometer using a 150 W CW Ozone-free xenon arc lamp as a source and a Photomultiplier R928P (spectral coverage 200 nm – 900 nm, cooled and stabilized) as the detector.

A Seven Compact digital pH-meter by Mettler Toledo was used for pH determinations. The pH electrode was an InLab Semi-Micro glass electrode. The pH meter was calibrated before each set of measurements using standard buffer solutions at pH 4.01, 7.00, and 10.01, ensuring accurate readings across the relevant pH range.

3.7.2 Synthesis of molecules and polymers

2,9-bis(2-(piperazin-1-yl)ethyl)anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetraone – piperPDI



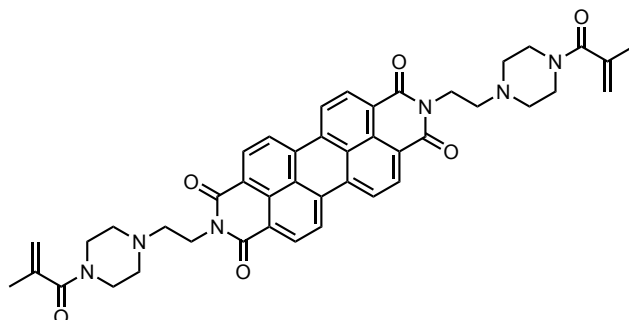
Briefly, 3,4,9,10-perylenetetracarboxylic dianhydride (392.3 mg, 1.00 mmol) was placed in a Schlenk tube equipped with a magnetic stirring bar together with 1-(2-aminoethyl)piperazine (5 mL, 38 mmol). The tube was sealed, subjected to four freeze–pump–thaw cycles, and refilled with argon for 30 min. The mixture was then heated to 160 °C and stirred for 22 h. After cooling to room temperature, the reaction crude was poured into a glass beaker containing dichloromethane (50 mL) and stirred for 30 min. The resulting suspension was filtered under reduced pressure, affording the product as a dark purple powder in quantitative yield (>95%).

^1H NMR (400 MHz, TFA-d): δ (ppm) = 8.82 (dd, J = 43.2, 8.1 Hz, 8H), 4.82 (t, J = 5.4 Hz, 4H), 4.46 (d, J = 13.1 Hz, 4H), 3.97 (m, J = 17.4, 6.7 Hz, 16H), 3.86 (m, J = 13.5, 4.9 Hz, 5H).

^{13}C NMR (101 MHz, TFA-d): δ (ppm) = 166.13, 136.46, 133.25, 129.29, 126.44, 124.36, 121.30, 49.42, 41.70.

ESI-MS (in H_2O + 1% v/v HCOOH): m/z (%) = 615.27 ($\text{M}^+ + 1$), 308.30 ($\text{M}^{2+} + 1$).

2,9-bis(2-(4-methacryloylpiperazin-1-yl)ethyl)anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetraone – piperDMA



PiperPDI (614.7 mg, 1.00 mmol) and triethylamine (1.39 mL, 10 mmol) were partially dissolved in anhydrous chloroform (20 mL) by sonication for 10 min. The solution was transferred to a round-bottom flask equipped with a magnetic stirrer and an addition funnel and cooled to 0 °C using an ice bath. Methacryloyl chloride (980 μ L, 10 mmol), dissolved in anhydrous chloroform (10 mL), was added dropwise over 20 min under stirring. During the addition, the reaction mixture gradually turned red, and the formation of white fumes (HCl) was observed. After completion of the addition, the reaction was stirred for 1 h at 0 °C and then allowed to warm to room temperature and stirred for an additional 24 h. The crude reaction mixture was poured into cold acetone (100 mL), resulting in precipitation of the product. The solid was collected by centrifugation and washed sequentially with acetone (2 $\times$ 30 mL) and water (2 $\times$ 30 mL). The final product was dried under vacuum overnight to afford a bright red powder in 79% yield.

^1H NMR (400 MHz, TFA- d): δ (ppm) = 8.88 (d, J = 8.3 Hz, 4H), 8.80 (d, J = 8.1 Hz, 4H), 5.47 (d, J = 1.9 Hz, 2H), 5.27 (s, J = 1.1 Hz, 2H), 4.90 (m, J = 15.5 Hz, 2H), 4.82 (t, J = 5.4 Hz, 4H), 4.41 (d, J = 15.1 Hz, 2H), 4.16 (d, J = 11.7 Hz, 4H), 3.88 (t, J = 5.3 Hz, 13H), 1.97 (s, 6H).

ESI-MS (in H_2O + 1% v/v HCOOH): m/z (%) = 751.32 ($\text{M}^+ + 1$), 376.17 ($\text{M}^{2+} + 1$).

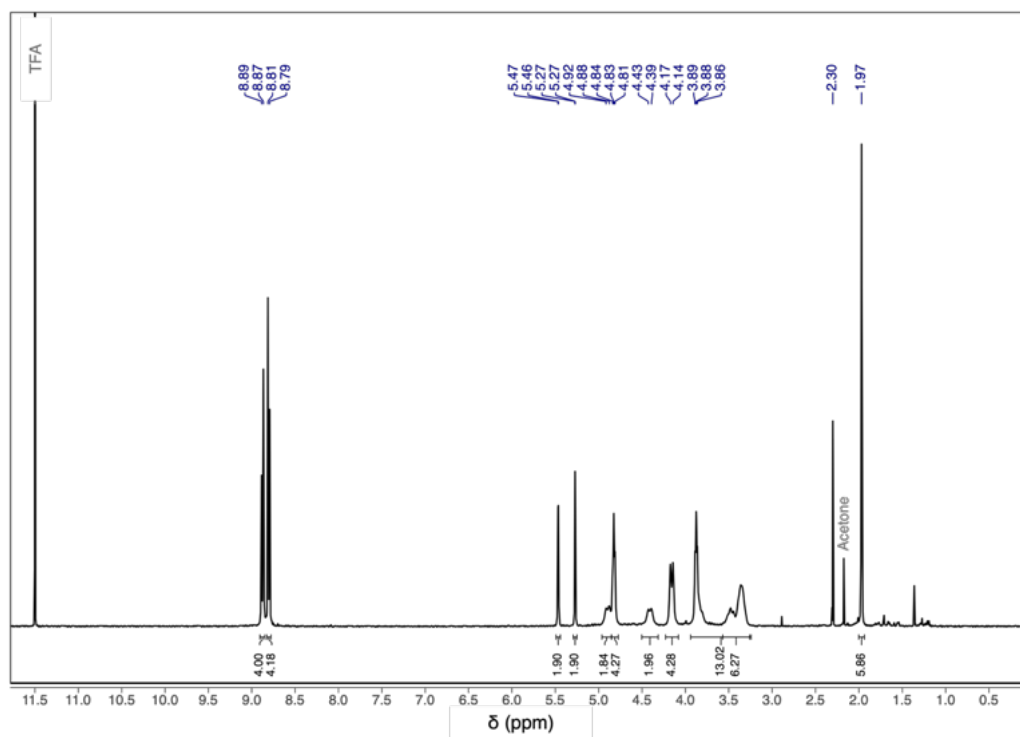


Figure S15 ¹H NMR spectrum of piperDMA acquired in TFA-d and referenced against residual TFA.

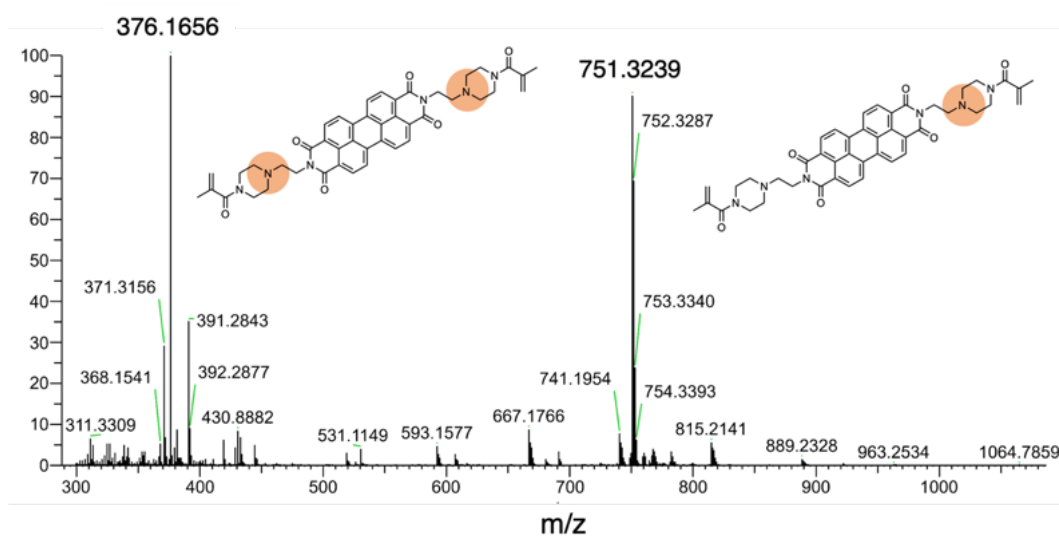
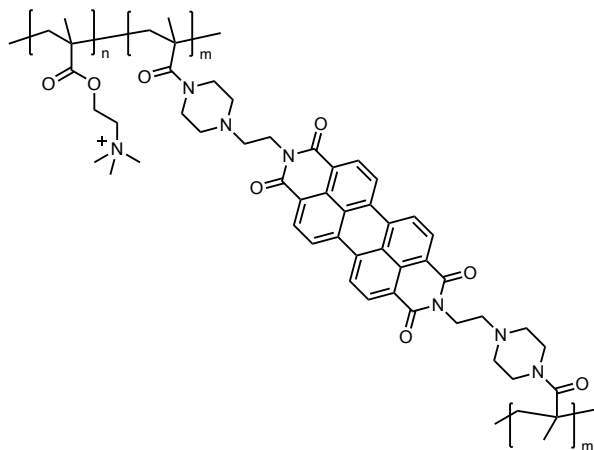


Figure S16 ESI-MS accurate mass spectrum for piperDMA in H₂O + 1% v/v HCOOH.

**Poly(2-(trimethylammoniummethyl methacrylate) chloride)-*co*-piperDMA –
PTMAEMA-*co*-piperDMA**



PTMAEMA-*co*-piperDMA was synthesized by free radical polymerization. 2-(methacryloyloxy)ethyl]trimethylammonium chloride (TMAEMA) and ACVA were used as purchased without purification. TMAEMA (207.7 mg, 1 mmol), piperDMA (7.5 mg, 0.01 mmol), and ACVA (1.16 mg, 4 μ mol) were dissolved in water (1 mL) in a vial equipped with a stirrer bar. Full solubilization was achieved by adding HCl 1 M. DMF (100 μ L) was added as an internal standard to assess the monomer conversion via ¹H-NMR. The vial was sealed, and the polymerization was carried out at 70 °C for 24 hrs under stirring. The polymer was isolated by precipitation from acetone as a crystalline red powder (yield = 74%). Conversion (¹H-NMR) 97%.

3.7.3 Experimental

A1 – Preparation of stable coacervate droplets. Stock solutions of each polymer were prepared in Milli-Q water. For molarity calculations, we referred to the molecular weights (Mw) of the monomers (PTMAEMA: 207.7 g mol⁻¹, ATP sodium salt: 110.2 g mol⁻¹, styrene sulfonate: 206.2 g mol⁻¹). The pH of the solutions was adjusted to 3 by the addition of either HCl (0.5 M) or NaOH (0.5 M).

Stable coacervate droplets were prepared by mixing 200 µL of a 10 mM PTMAEMA-co-piperDMA stock solution with 200 µL of a 10 mM ATP stock solution in a 2 mL Eppendorf tube. Upon mixing, the solution became cloudy, indicating the formation of a coacervate dispersion. The sample was gently homogenized by manual pipette mixing and subsequently centrifuged at 2000 rpm for 5 min to separate the dense coacervate phase from the dilute supernatant. After centrifugation, the supernatant was carefully removed and replaced with 1 mL of Milli-Q water. The sample was then vortexed for 2 min to fully redisperse the dense phase, resulting in a slightly milky dispersion corresponding to a stable population of coacervate droplets.

For the preparation of enzyme-loaded coacervate droplets, the same procedure was followed, with the addition of 10 µL of a 1 mg mL⁻¹ urease solution to the PTMAEMA-co-piperDMA solution prior to mixing with ATP.

A2 – Enzyme labelling. 20 mg of urease were dissolved in 7.4 mL of sodium carbonate buffer (pH 8.5, 0.1 M). Fluorescein isothiocyanate (FITC) was dissolved in DMSO to yield 1 mg mL⁻¹ stock solution. 200 µL of the fluorescent tag solution was added to the enzyme aqueous solution, and the mixture was stirred overnight at 4 °C. The product was purified by dialysis, lyophilized, and stored as a solid at -20 °C.

A3 – Microscopy imaging of PTMAEMA-co-piperDMA/ATP coacervate droplets. Once prepared as reported in A1, 100 µL stable coacervate droplet dispersion were transferred to a microscopy plate, either a Petri dish or a multi-wheel plate. To further prevent the sedimentation of the droplets, right before use, the

microscopy glasses were washed with EtOH, 1 M NaOH, 1 M HCl, and then coated with a 2% w/w solution of PVP in water, for at least 5 hrs. At the end, one last rinse with Milli-Q water was performed to eliminate the excess PVP.

To perform imaging at different pH values, 100 μ L of a 10 mM solution of the proper buffer (3-5 citrate, 6-7 phosphate, 8-9 tris) were added. The images were then taken after 10 minutes to allow complete diffusion inside the droplets.

A4 – Titration of piperDMA and PTMAEMA-co-piperDMA. For pH-dependent spectroscopic studies, a 0.1 mg mL⁻¹ aqueous solution of the PET-based molecule (either piperDMA or PTMAEMA-co-piperDMA) was prepared in Milli-Q water. The initial pH was adjusted to 2.5 by the addition of 1 M HCl.

Potentiometric titrations were first carried out by incremental addition of 50 mM NaOH to 3 mL of the stock solution while continuously monitoring the pH using a calibrated pH meter. Once the relationship between added base and pH variation was established, identical NaOH additions were reproduced for spectroscopic measurements. UV-vis absorption spectra were recorded in the range 200-700 nm. Fluorescence measurements were performed by exciting the samples at 501 nm and collecting emission spectra from 510 to 700 nm. These experiments allowed direct correlation between pH variations and the optical response of the PET-based systems.

The Henderson-Hasselbach equation was used to calculate the apparent pK_a value of piperDMA and PTMAEMA-co-piperDMA:

$$\log \frac{I_{F \max} - I_F}{I_F - I_{F \min}} = pH - pK_a \quad \text{(Equation S1)}$$

A5 – Fluorescence recovery after photobleaching (FRAP). FRAP experiments were performed on an Olympus FV3000 confocal laser scanning microscope utilizing a 60x oil immersion objective lens. Fluorescence recovery was recorded for 30 minutes with an acquisition rate of one frame every 10 s. To obtain a pre-bleaching reference, one image was taken before bleaching. Bleaching was performed using a 488 nm laser

at 50% intensity for 2 s in a circular area of approximately 1 μm in radius, with a scanning speed of 2 μs /pixel. FRAP analysis was carried out using the latest version of ImageJ software.

A6 – Urea-urease reaction network within coacervate droplets. Enzyme kinetics experiments were performed in a PVP-coated multi-wheel plate. In each sample wheel, 100 μL of urease-loaded coacervate droplets were added. After deposition of the droplets at the bottom, 100 μL of Milli-Q were added. Finally, to start the experiment, 100 μL of a urea stock solution (10, 25, 50, and 100 mM) were added. Imaging started before the last addition to better visualize the kinetics. Experiments were recorded for 20 minutes with an acquisition rate of one frame every 20 s. For the experiments with a buffer, it was added instead of water as a second addition in the wheel. Images were analyzed using the latest version of ImageJ software.

A7 – Fluorescence studies for enzyme kinetics. To correlate fluorescence intensity of the droplets with the enzyme kinetics, the Michaelis-Menten equation was linearized using a Lineweaver-Burk plot to better estimate the parameters. Initial reaction rates were obtained from the slope of the fluorescence time traces at short reaction times, where the signal decrease was linear. The absolute value of the initial fluorescence quenching rate ($|\text{dF}/\text{dt}|$) was taken as a proxy for the apparent reaction velocity. Effective substrate concentrations were corrected to account for dilution effects in the measurement volume. Apparent kinetic parameters were estimated by fitting the initial rates to the Michaelis-Menten equation:

$$v = \frac{v_{\max} \times [S]}{K_M + [S]} \quad (\text{Equation S2})$$

Lineweaver–Burk plots were constructed according to:

$$\frac{1}{v} = \frac{K_M}{v_{\max}} \times \frac{1}{[S]} + \frac{1}{v_{\max}} \quad (\text{Equation S3})$$

and the slope and intercept were obtained by least-squares linear regression. Apparent values of K_M and v_{max} were calculated from the fitted parameters as $K_M = slope|v_{max}$ and $v_{max} = 1/intercept$. All kinetic parameters are reported as apparent values reflecting the coupled enzymatic reaction and pH-sensitive fluorescence readout.

3.7.4 Supplementary figures

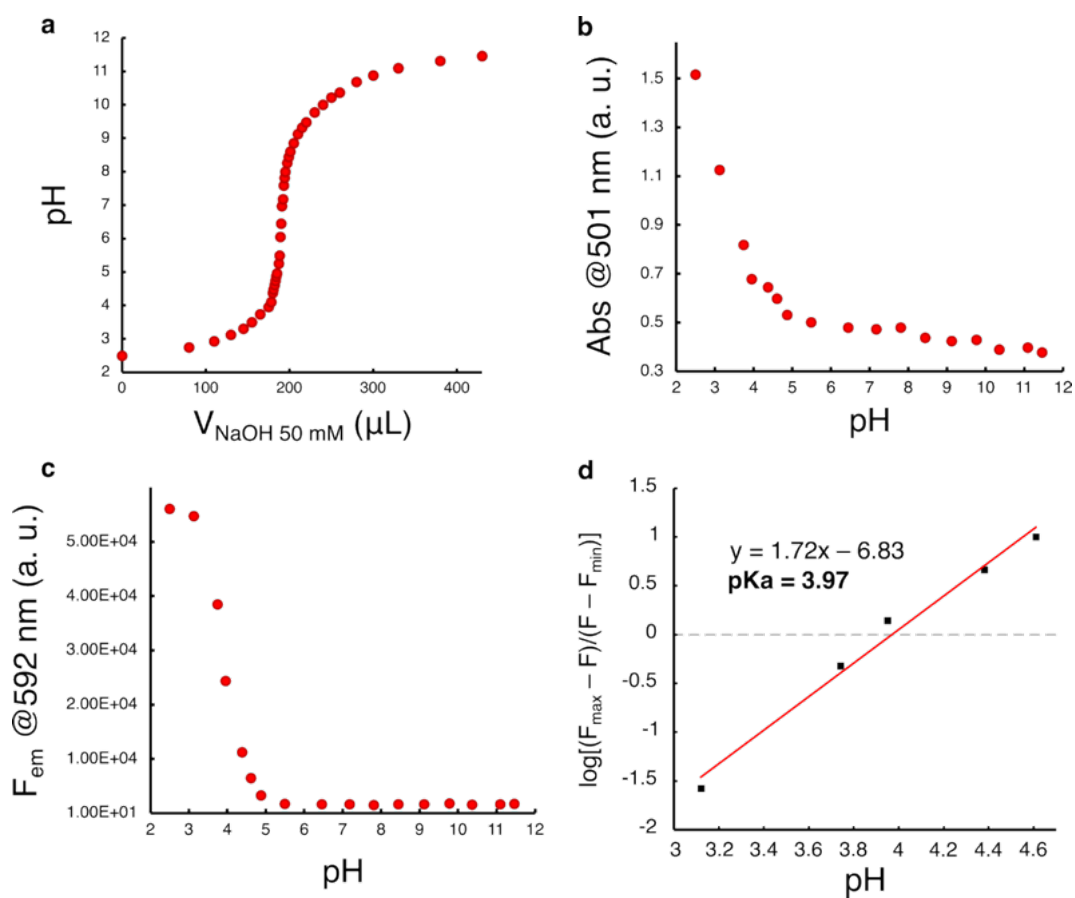


Figure S17 PiperPET pH titrations. **a**, pH variation as a function of added 50 mM NaOH. **b**, Changes in UV-vis absorbance with increasing pH, showing a clear ON-OFF transition around pH 5. **c**, Fluorescence emission intensity as a function of pH, also displaying an ON-OFF switch near pH 5. **d**, Henderson-Hasselbach plot for piperPET yielding an apparent pKa at 3.97.

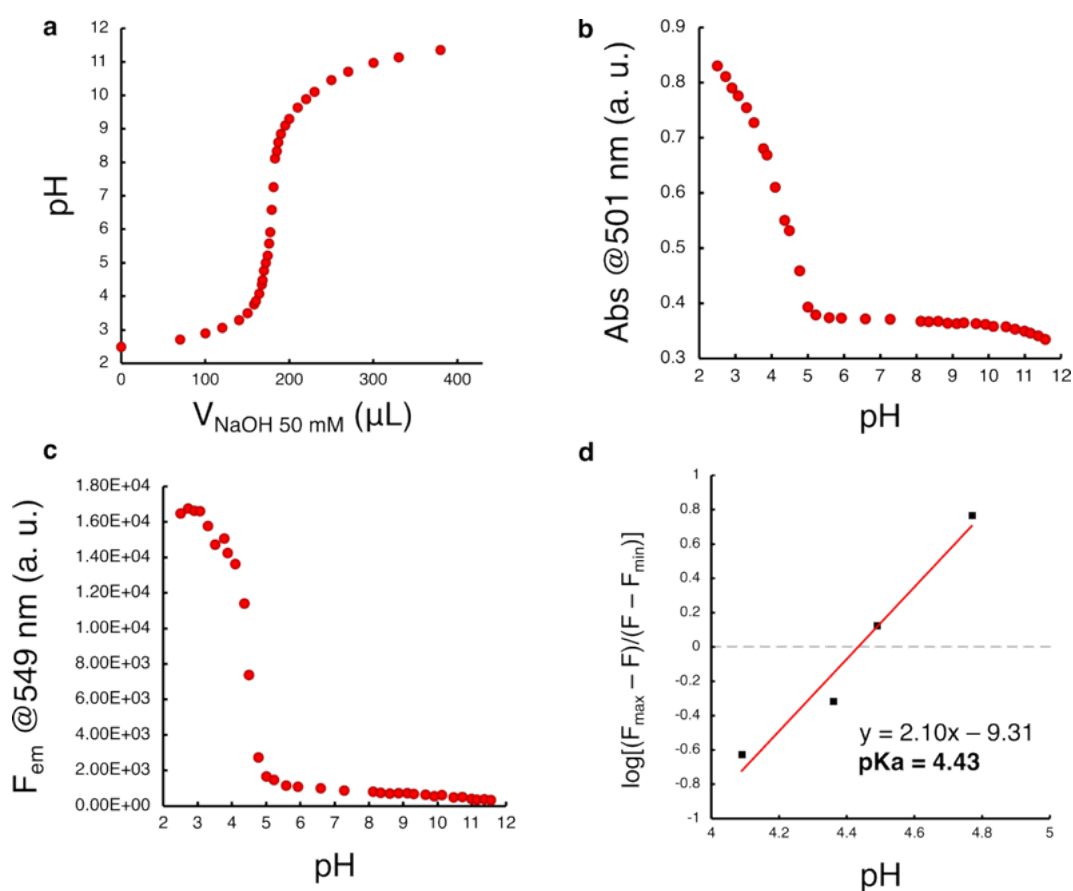


Figure S18 PTMAEMA-co-piperPET pH titrations. **a**, pH variation as a function of added 50 mM NaOH. **b**, Changes in UV-vis absorbance with increasing pH, showing a clear ON-OFF transition around pH 5. **c**, Fluorescence emission intensity as a function of pH, also displaying an ON-OFF switch near pH 5. **d**, Henderson-Hasselbach plot for the polymer yielding an apparent pK_a at 4.43.

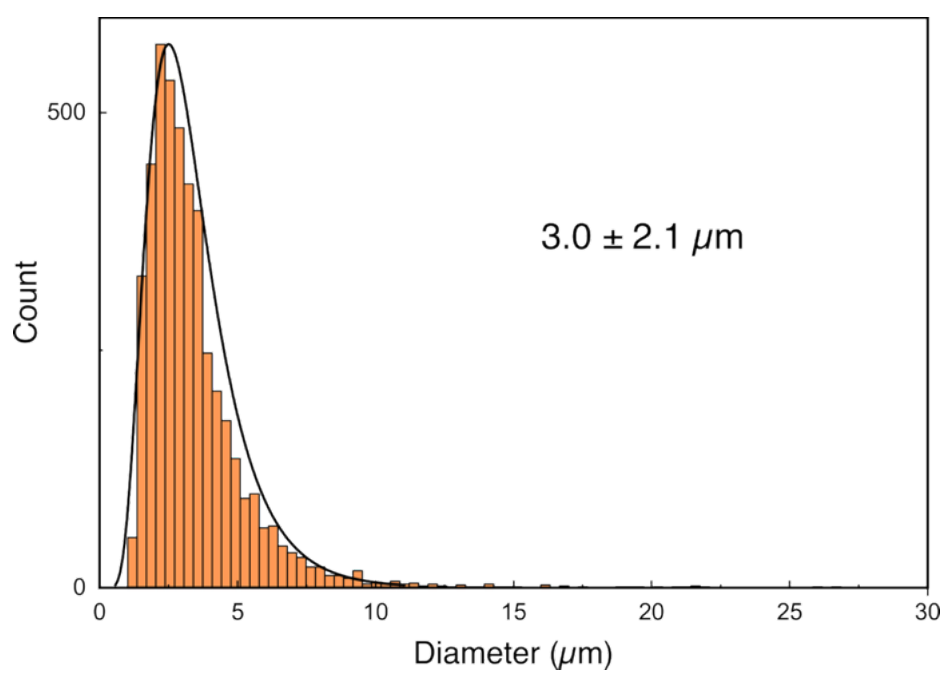


Figure S19 Histogram showing the size distribution of PET-based coacervate droplets. The sample is polydisperse, with a large number of small droplets ($< 5 \mu\text{m}$). The mean diameter was $3.0 \pm 2.1 \mu\text{m}$.

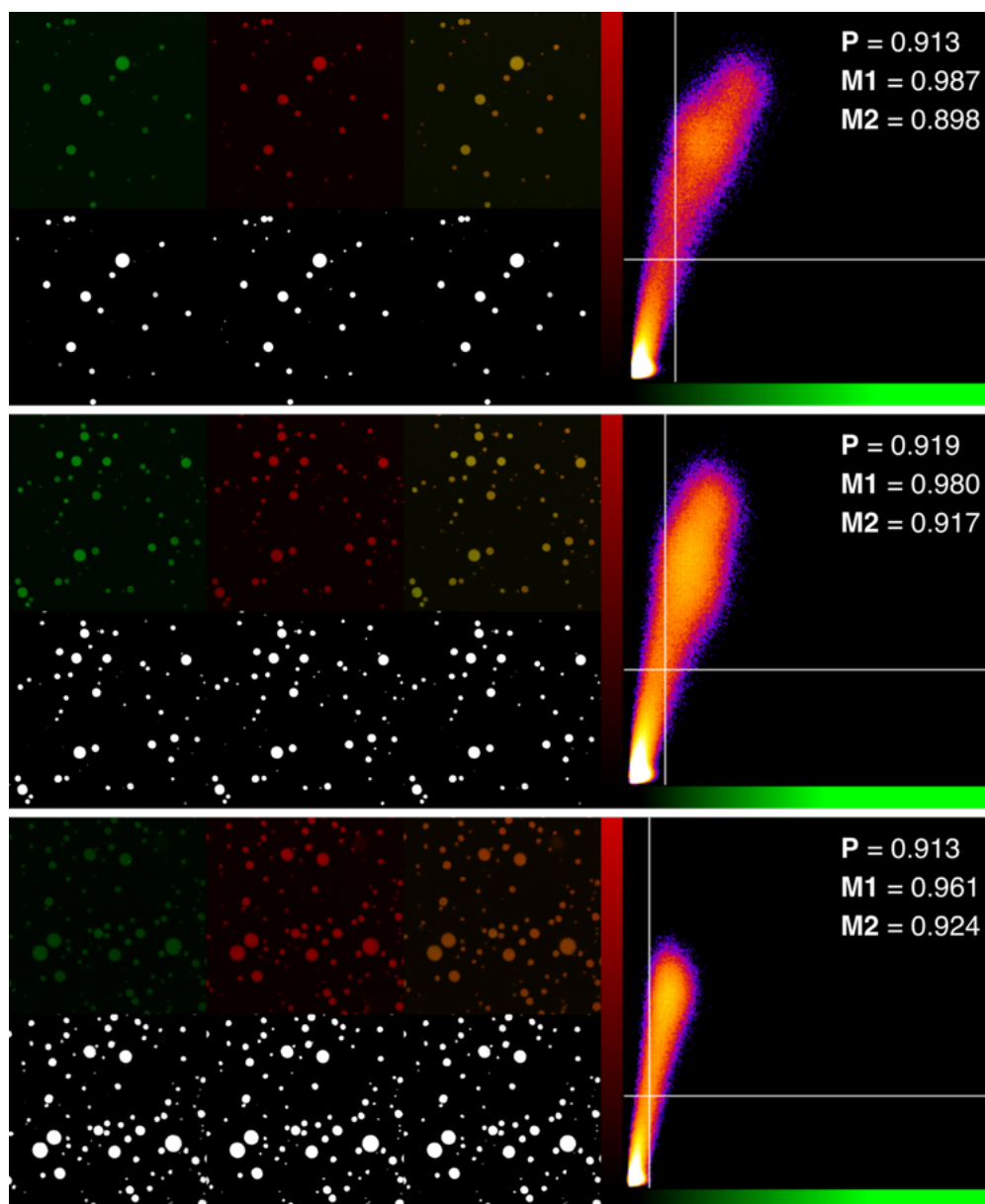


Figure S20 Colocalization studies of three different samples of FITC-tagged urease-loaded coacervate droplets. Red, green, and merged channels of a confocal laser scanning microscopy image are reported with corresponding binary thresholded images. They show high pixel overlap. On the right is reported the colocalization scatterplot of the individual pixels from the paired images, with white lines indicating threshold levels for both channels. Most pixels above the threshold values are aligned in the top-right quadrant, suggesting nearly complete colocalization. In the scatterplot are reported the Pearson's correlation coefficients (P) and Mander's coefficients (M1, M2). Both Pearson's and Mander's coefficient values approach 1, indicating that nearly all pixels of the two thresholded channels overlap, demonstrating high colocalization. Colocalization parameters were quantified using the BIOP JACoP plugin in ImageJ, applying an automatic threshold set by the Otsu method.

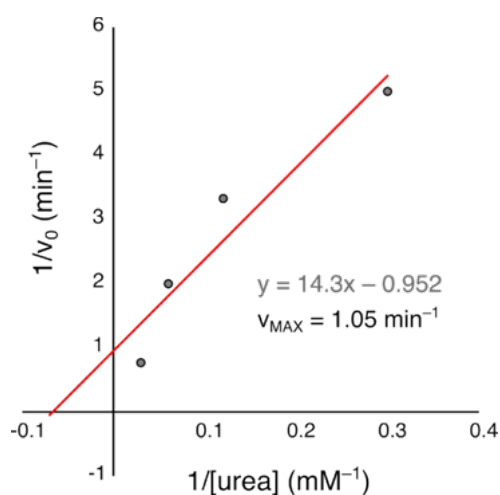


Figure S21 Lineweaver-Burk plot of the apparent initial reaction rates obtained from fluorescence quenching measurements. The reciprocal of the apparent initial rate ($1/v$) is plotted as a function of the reciprocal substrate concentration ($1/[\text{urea}]$), using effective urea concentrations corrected for dilution. Initial rates were extracted from the linear regime of the fluorescence time traces. The solid line represents a least-squares linear fit used to estimate apparent kinetic parameters K_M and v_{MAX} . Deviations at higher substrate concentrations (lower $1/[\text{urea}]$) reflect non-linear pH transduction effects of the sensing system rather than intrinsic enzymatic kinetics.

4 COACERVATE VESICLES AS PHOTOCATALYTIC PROTOCELLS

The experiments, findings, and conclusions presented in this chapter constitute core scientific contributions to the PLANKT-ON project (EIC Grant agreement ID: 101099192, DOI: <https://doi.org/10.3030/101099192>). These results have been included in the official project reports and presented during progress meetings, and will contribute to forthcoming publications and patent applications.

ICP-AES measurements were performed in collaboration with Prof. M. Crosera and Dr. G. Marussi, University of Trieste, Trieste, IT. Fourier-transform hyperspectral microscopy analyses were performed by Prof. Margherita Maiuri and Dr. Mattia Russo, Politecnico di Milano, Milan, IT.

We gratefully acknowledge Prof. Marcella Bonchio and her research group, including Dr. Francesco Rigodanza, Dr. Thomas Gobbato, Gianpaolo Marraffa, and Giulia Erichiello, for the synthesis and characterization of the Ru₄POM and PBI-TEG starting materials, as well as for the valuable and constructive discussions that contributed significantly to the development of this project over the years.

4.1 INTRODUCTION

Natural photosynthesis in plants and plankton is an intricate process that involves a sequence of highly coordinated chemical and physical reactions.¹ It begins with water oxidation during the light phase and proceeds with the reductive fixation of CO₂ to generate energy-rich glucose during the dark phase.² Crucially, photosynthesis operates under continuous energy input and far from thermodynamic equilibrium, enabling energy transduction and chemical transformations that would not occur spontaneously under equilibrium conditions.^{3,4} This sustained non-equilibrium operation is a defining feature of biological energy conversion and underpins the remarkable efficiency and adaptability of photosynthetic systems.

Phytoplankton are aquatic photosynthetic monocellular organisms that represent only about 2% of the global plant biomass on Earth, but are responsible for approximately 40% of global photosynthetic activity and carbon fixation.^{5,6} Geological and geochemical evidence indicates that early protocellular phytoplankton significantly contributed to the rise of atmospheric oxygen, reaching levels comparable to those of today as early as 2.2 billion years ago.⁷ This biologically driven transformation marks a critical transition in Earth's geochemical evolution and profoundly influenced the subsequent emergence and diversification of complex life.

Inspired by these natural systems, the PLANKT-ON project aims to develop an artificial plankton cell composed of two distinct functional organelles capable of producing oxygen and formic acid from water using solar light (Figure 4.1).⁸ This chapter focuses on the development of a coacervate-based artificial organelle designed to perform photoinduced water oxidation. The design, characterization, and performance of this system are discussed in detail, highlighting its relevance as a minimal, non-equilibrium model for solar light-driven oxygen evolution in artificial protocells.

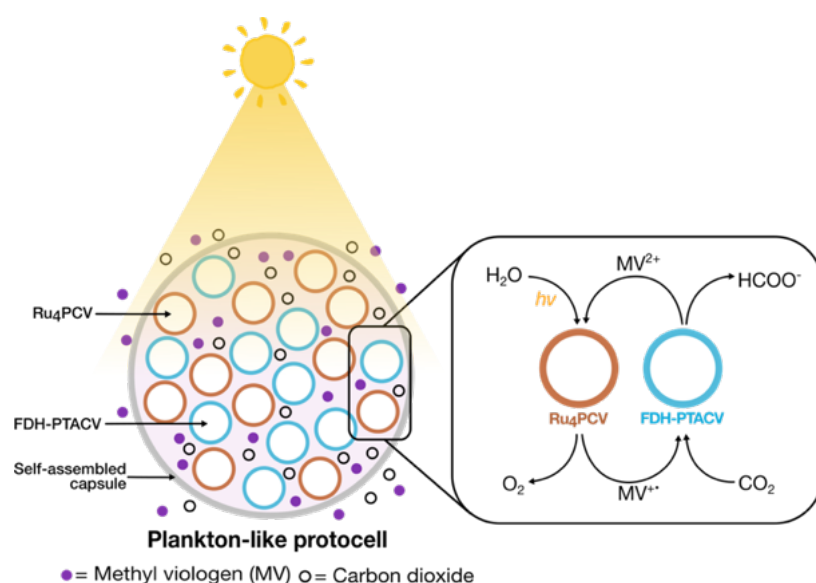


Figure 4.1 Schematic representation of a plankton-like protocell capable of converting water into oxygen and formic acid under light irradiation. The first step of the photocatalytic cycle is carried out by Ru₄PCVs and consists of a photoinduced water oxidation process.

4.1.1 Polyoxometalate coacervate vesicles

Polyoxometalates (POMs) are well-studied inorganic metal oxyanion clusters based on the assembly of MO_x units, where M is an early transition metal such as tungsten, molybdenum, or vanadium, and x ranges between 4 and 7.⁹ These clusters can act as multidentate ligands and can introduce magnetic character into the systems in which they are incorporated. Because of their high negative charge, POMs readily form complexes with metal cations, commonly referred to as heteroatoms. In their fully assembled form, POMs that include heteroatoms constitute a versatile class of inorganic compounds with notable properties, including strong acidity, rich redox behavior, high conductivity, and remarkable structural diversity.¹⁰ These characteristics arise from several features of their molecular architecture. POMs often possess a high degree of structural symmetry, and their redox-active metal centers can modulate electron distribution, which in turn adjusts intermolecular interactions. Heteroatoms can further influence catalytic performance and stability. Their broad solubility in many solvents, particularly water, allows them to be introduced into a wide range of chemical

environments and incorporated into both solid-state and solution-based systems.¹¹ Importantly, POM-based materials can exhibit cooperative properties, including non-linear optical responses and even superconductivity. The ability to tune these behaviors by modifying composition and structure has made POMs valuable components in the design of advanced functional materials.^{12,13} As a result, POM-containing systems have found applications across many fields, ranging from catalysis and energy conversion to host–guest chemistry and biomedical research.^{14,15}

As introduced in Chapter 1.3, Williams *et al.* reported in 2014 that a simple negatively charged polyoxometalate can induce the formation of a new class of coacervate vesicles that retain the sponge-like internal environment of coacervates while exhibiting enhanced stability.¹⁶ These vesicles were generated through a spontaneous one-step vacuolization of poly(diallyldimethylammonium chloride) (PDDA)/adenosine 5'-triphosphate (ATP) coacervate microdroplets triggered by the addition of phosphotungstic acid (PTA). Following the addition of PTA, strong electrostatic interactions between the positively charged PDDA chains and the PTA anions lead to deformation of the original coacervate droplet and to the establishment of an osmotic pressure with a resulting water influx into the structure. The resulting polyoxometalate coacervate vesicle (PCV) displays a three-tiered organization composed of a thin PTA–PDDA membrane, a PDDA–ATP coacervate sub-membrane, and an internal aqueous lumen.

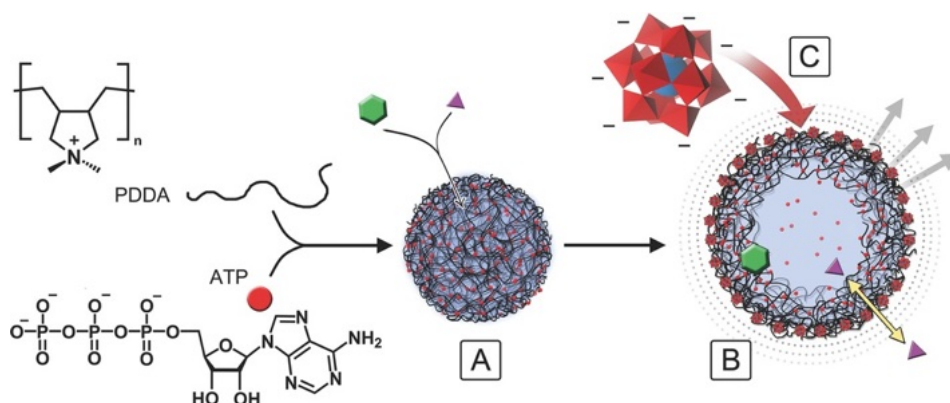


Figure 4.2 Schematic formation of PCVs. Coacervate microdroplets (A) can be obtained by mixing polycationic PDDA (poly(diallyldimethylammonium chloride) and polyanionic ATP (adenosine 5'-

triphosphate). These molecularly crowded coacervate microdroplets are transformed into PCVs (B) by the addition of PTA (phosphotungstic acid) (C). PCVs retain the property of coacervates to sequester molecules (green and purple figures) into their coacervate membrane. Image reproduced with permission from reference [16]. Copyright 2014 Wiley VCH Verlag GmbH & Co. KGaA.

This type of protocell exhibits strong birefringence under cross-polarized light, which indicates the presence of an ordered and stabilizing membrane architecture.¹⁷ Furthermore, PCVs show good stability across a range of pH values and temperatures, demonstrating that they form robust and durable structures suitable for further engineering.

4.1.2 Ru₄POM coacervate vesicles

Building on this model, Gobbo *et al.* developed PCVs containing both a catalytic polyoxometalate and the inactive PTA.¹⁸ To achieve this, they exploited a system previously reported by Prof. Bonchio's group at the University of Padova, which features a tetraruthenium(IV)- μ -oxo core embedded between two divacant γ -decatungstosilicate ligands, forming the negatively charged complex $[\text{Ru}_4(\mu\text{-O})_4(\mu\text{-OH})_2(\text{H}_2\text{O})_4(\gamma\text{-SiW}_{10}\text{O}_{36})_2]^{10-}$, or Ru₄POM (Figure 4.3).¹⁹ Ru₄POM has been used as a catalyst for water oxidation in electrocatalytic conditions,^{20,21} and more importantly in photo-electrocatalytic systems when coupled with a suitable photosensitizer such as Ru(II) polypyridines. These photosensitizers capture incoming light and enable Ru₄POM to perform efficient hole-scavenging reactions.²²

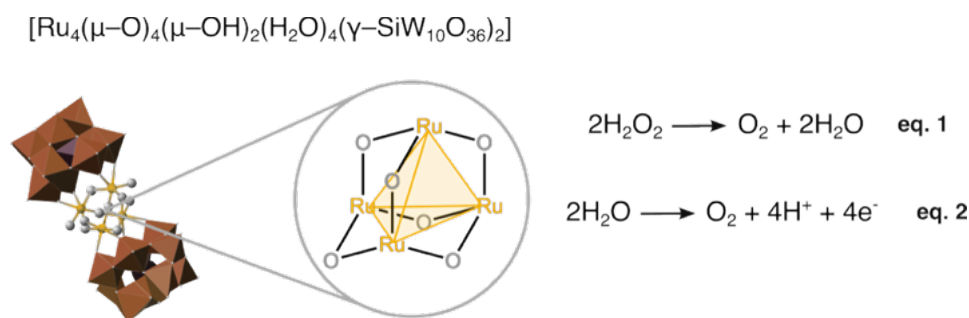


Figure 4.3 Schematic structure of Ru₄POM. The structure is composed by two γ - decatungstosilicate (in brown) surrounding a tetraruthenium(IV)- μ -oxo core. The core presents an adamantane-like arrangement, with 4 ruthenium atoms at the vertices of a tetrahedron, surrounded by 6 oxygen atoms. On the right are reported the reactions that it can perform: eq. 1 H_2O_2 disproportion (catalase-like activity), and eq. 2 water oxidation.

Owing to its water solubility and highly negative charge, Ru₄POM could be combined with PTA, which functions as a templating agent during PCV formation. This process converts PDDA/ATP coacervate droplets into Ru₄PCVs. Stable and well-structured vesicles were obtained only at a 1:7 molar ratio between Ru₄POM and PTA, indicating that the catalytic component must remain a minority species in the membrane. In this first pioneering study, which represents the earliest example of catalytic functionalization of a PCV, Gobbo *et al.* demonstrated that the resulting protocells can perform catalase-like activity. Using the oxidative ruthenium core of the polyoxometalate, the Ru₄PCVs converted hydrogen peroxide into water and oxygen. They also showed that this activity could be interfaced with enzyme-loaded coacervate vesicles, forming a simple communicative artificial system. This behavior provides a conceptual foundation for the long-term objectives of the PLANKT-ON project.

More recently, Ru₄PCVs have been engineered to perform not only catalase-like reactions but also photoinduced water oxidation. Rebasa-Vallverdu *et al.* reported that, similarly to the behavior of Ru₄POM in homogeneous solution, the addition of a photosensitizer such as tris(2,2'-bipyridine)ruthenium(II) ($[\text{Ru}(\text{bpy})_3]^{2+}$), together with an electron acceptor like sodium persulfate ($\text{Na}_2\text{S}_2\text{O}_8$) allowed the formation of a photosynthetic unit capable of producing oxygen under white light irradiation.²³ These photocatalytic protocells can operate continuously for several hours, possess robust

and easily handled membranes, and can be reused even after long-term storage periods exceeding 12 to 24 months. However, their formation requires a low content of catalytic Ru₄POM in the membrane (1:7 relative to non-catalytic PTA), and their photocatalytic activity depends strongly on the presence of high concentrations of [Ru(bpy)₃]²⁺ in solution. These two aspects are not fully compatible with the industrial goals of the PLANKT-ON project, and strategies to overcome these limitations will be discussed in the following paragraphs.

4.1.3 Artificial quantasome for photoinduced water oxidation

In living organisms, solar light-induced water splitting is carried out by Photosystem II, a highly sophisticated enzymatic complex composed of multiple chromophoric light-harvesting antennae arranged around a central catalytic reaction center.²⁴ Before the detailed structure and function of Photosystem II were fully elucidated, Park and Calvin proposed the existence of a nanoscale photosynthetic unit termed the quantasome, envisioned as a self-contained light-harvesting and catalytic module.²⁵ This concept has continued to inspire the design of artificial assemblies that combine light absorption and catalytic activity within a single, integrated architecture.²⁶ Building on this idea, Bonchio *et al.* recently developed an artificial quantasome-like system (QS) in which the catalytic Ru₄POM is electrostatically coupled to a light-harvesting antenna composed of five charged perylenebisimide units (PBI-TEG).^{27,28} This synthetic assembly efficiently captures visible light and promotes the oxidation of water. However, owing to its hydrophobic character and poor solubility in aqueous media, this artificial quantasome has so far been employed primarily in photoelectrocatalytic configurations, typically immobilized on transparent electrodes.²⁹ The integration of such a self-standing photosynthetic unit into a soft, aqueous-compatible material would represent a significant advance in the field. This approach could open new opportunities for artificial photosynthesis, green energy storage, and the development of protocellular systems capable of solar-to-chemical energy conversion in water.

4.2 SYNTHESIS AND CHARACTERIZATION OF 100% Ru₄PCVs

To address the low catalytic loading of Ru₄POM in the first generation of Ru₄PCVs, which relied on an excess of non-catalytic phosphotungstic acid (PTA) as the anionic component, ATP was replaced with low-molecular-weight poly(acrylic acid) (PAA) as the negatively charged polyelectrolyte. 500 μ L of a 20 mM solution of PAA was added to 500 μ L of a stirring solution of polydiallyldimethylammonium chloride (PDDA) at pH 6.5 to form coacervate microdroplets. Then 100 μ L of Ru₄POM 6 mM was rapidly added, and the solution was kept stirred for an additional 30 s (Figure 4.4).

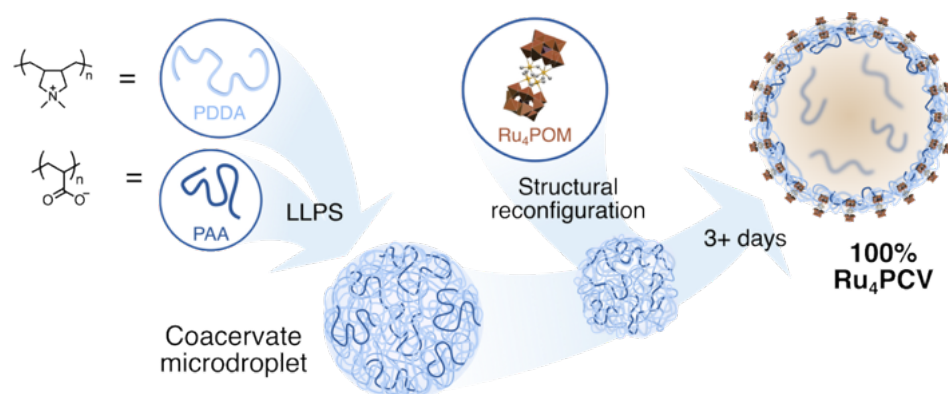


Figure 4.4 Scheme describing the electrostatic assembly of 100% Ru₄PCVs. The addition of Ru₄POM to a dispersion of PDDA/PAA coacervate microdroplets first produced deformed microdroplets that gradually organized into membranized vesicular structures.

Unlike ATP, which was previously observed to diffuse out of the coacervate phase during droplet-to-vesicle reconfiguration, thereby preventing vesicle formation when Ru₄POM was used as the sole morphogenic species (Figure S22),^{16,30} PAA forms stronger electrostatic interactions with PDDA. As a result, the addition of Ru₄POM led to the formation of compressed and deformed coacervate microdroplets. Over the first 3 hours after preparation, these structures developed an internal aqueous lumen and progressively reconfigured into PCVs over 3 to 7 days. This transformation was

attributed to a gradual inward water flux driven by osmotic pressure differences across the coacervate membrane (Figure 4.5a,b). The resulting PCVs, composed exclusively of Ru₄POM at the membrane and hereafter referred to as 100% Ru₄PCVs, exhibited a mean diameter of $25 \pm 10 \mu\text{m}$, and presented a strong birefringence pattern indicating the presence of a highly ordered membrane structure (Figure 4.5c,d).

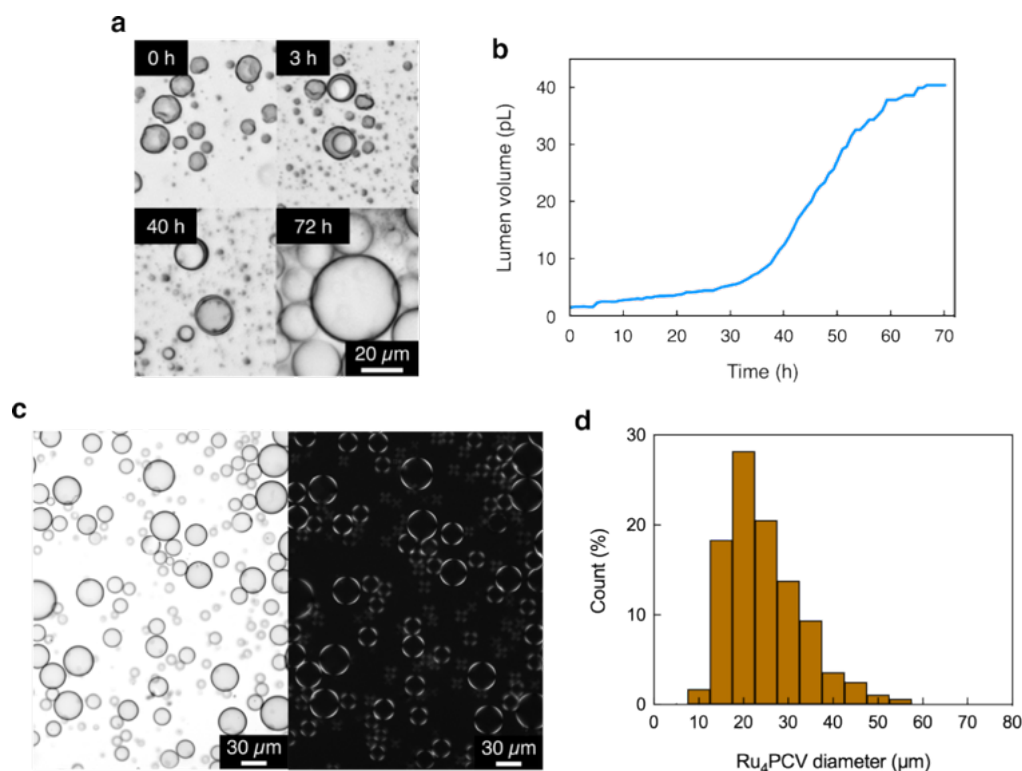


Figure 4.5 **a**, Time-dependent brightfield microscopy images showing the reconfiguration of coacervate microdroplets into Ru₄PCVs. **b**, Graph showing the increase in volume of a representative 100% Ru₄PCV over time. **c**, Brightfield and cross-polarized microscopy images of a Ru₄PCVs population. **d**, Size distribution histogram of 100% Ru₄PCVs.

The structural robustness of the organized membrane enabled lyophilization of the 100% Ru₄PCVs without loss of vesicular integrity. Each batch yielded a dry mass of $1.5 \pm 0.5 \text{ mg}$ after freeze-drying. Upon redispersion in water, the vesicles retained their original morphology and birefringence (Figure S23). The Ru₄POM content within the vesicles was quantified by inductively coupled plasma atomic emission spectrometry (ICP-AES). Each batch contained $43 \pm 7 \mu\text{g}$ of Ru₄POM, corresponding

to approximately 7.5 nmol and representing about 3 percent by weight of the total vesicle mass.

4.3 Ru₄PCVs AS PHOTOCATALYTIC PROTOCELLS

In a typical photocatalytic experiment, a batch of Ru₄PCVs was dispersed in the presence of [Ru(bpy)₃]²⁺ (40 µL, 50 mM) as the photosensitizer and sodium persulfate (100 µL, 100 mM) as the sacrificial electron acceptor in a Na₂SiF₆/NaHCO₃ buffer solution of (12 mM, pH 5.6), within a reactor vessel. The dispersion was continuously stirred at 1200 rpm to prevent diffusion limitations arising from vesicle sedimentation. Upon irradiation with white light (442 µW, 2.78 mW cm⁻²), immediate dioxygen evolution was observed. Oxygen production was monitored for 15 minutes using an oxygen probe. These experimental conditions were previously optimized for Ru₄POM/PTA/PDDA/ATP coacervate vesicles reported earlier.²³

Under these conditions, 100% Ru₄PCVs exhibited clear photocatalytic activity toward light-driven water oxidation, producing approximately 50 nmol of dioxygen within the first 15 minutes of irradiation (Figure 4.6a). However, the overall activity was lower than that of the previous Ru₄PCV generation. The initial oxygen evolution rates were $(3.83 \pm 1.39) \times 10^{-4}$ nmol s⁻¹ for the PTA/Ru₄POM/PDDA/ATP PCVs, and $(1.51 \pm 0.31) \times 10^{-4}$ nmol s⁻¹ for the 100% Ru₄PCVs (Figure 4.6b). This difference is notable given that the Ru₄POM content, as determined by ICP-AES, was lower in the former system (15 ± 5 µg) than in the latter (43 ± 7 µg). The reduced activity of the 100% Ru₄PCVs may be attributed to decreased membrane permeability resulting from the denser polymer network and the higher Ru₄POM loading. Moreover, in this system Ru₄POM fulfils both structural and catalytic roles, whereas in the previous architecture, PTA primarily contributed to self-assembly.

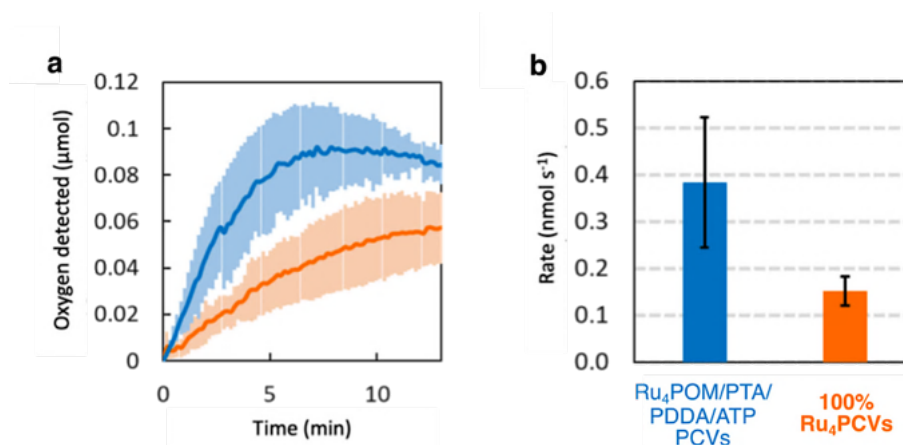


Figure 4.6 a, Graph showing the amount of dioxygen produced over time for the two systems. The blue curve and error bands refer to the Ru₄POM/PTA/PDDA/ATP PCVs, while the orange ones refer to the 100% Ru₄PCVs. **b**, Initial rate for the photocatalytic production of O₂ in the first two minutes of reaction.

From the perspective of the PLANKT-ON project, which aims to develop an industrially viable artificial protocellular system, the comparable photocatalytic performance achieved with a simplified composition and reduced auxiliary components represents a significant advance. Moreover, as discussed in the following section, the structural role of Ru₄POM in these vesicles has been critical for the development of a self-standing antenna-catalyst architecture within a protocellular framework.

4.4 QUANTASOME-PCVs

Incorporating the photosensitizer directly within the PCV architecture, rather than supplying it externally in the bulk solution as [Ru(bpy)₃]²⁺, would represent a substantial improvement in system integration and functional autonomy. To this end, we investigated the direct incorporation of the quantasome complex (QS), in which five PBI-TEG antenna units are electrostatically associated with a Ru₄POM catalytic core, into coacervates and PCVs. However, direct integration of the QS proved challenging due to its pronounced tendency to aggregate and precipitate in aqueous

media. While coacervates and PCVs efficiently sequester soluble macromolecules, they are unable to accommodate micrometer-scale aggregates, resulting in poor encapsulation efficiency and reduced structural stability (Figure S24).^{31,32}

To address this limitation, we adopted a stepwise strategy in which the water-soluble PBI-TEG photosensitizer was first incorporated into PDDA/PAA coacervate microdroplets, followed by an attempt to induce the *in situ* formation of quantasome-containing PCVs. As an initial step, the sequestration behavior of PBI-TEG within PDDA/PAA coacervates was examined. PDDA (500 μ L, 10 mM, pH 6.5) was mixed with PBI-TEG (50 μ L, 5 mM), followed by the addition of PAA (550 μ L, 20 mM, pH 6.5) to induce coacervate formation. Brightfield and fluorescence microscopy analysis revealed a homogeneous distribution of the red-emissive PBI-TEG within the coacervate phase as well as in the surrounding aqueous medium, with no evidence of aggregation (Figure S25). Building on these results, PBI-loaded PCVs were prepared using either PTA as a non-catalytic polyoxometalate control, which does not form QS assemblies, or Ru₄POM as the morphogenic component capable of QS formation (Figure 4.7).

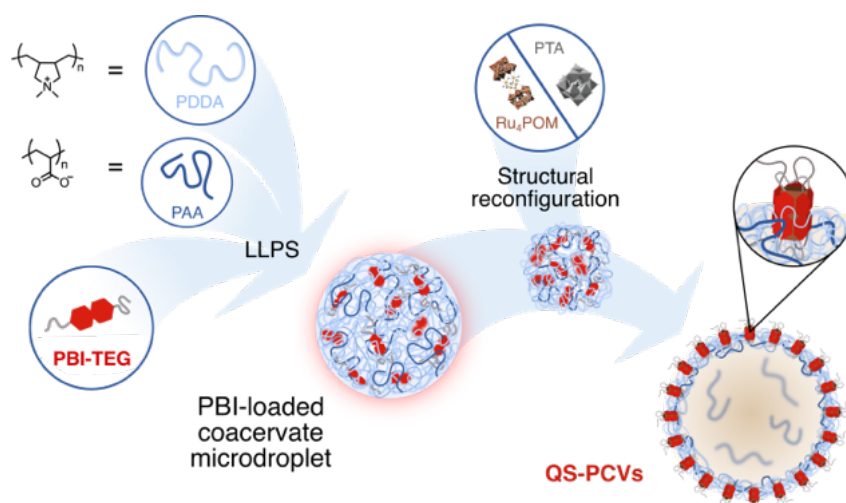


Figure 4.7 Scheme illustrating the electrostatic assembly of QS-PCVs or PBI-loaded PTA PCVs. The addition of Ru₄POM to a dispersion of PDDA/PBI-TEG/PAA coacervate microdroplets could both trigger the structural reconfiguration and form quantasome nanosystem on the vesicle membrane.

When PTA was used to induce the coacervate-to-vesicle transition, red-fluorescent PCVs were expected due to the absence of electronic interactions between PBI-TEG and the non-catalytic POM. Conversely, when Ru₄POM was employed, fluorescence quenching was anticipated as a result of either QS formation or energy transfer between PBI-TEG and the catalytic cluster. To evaluate these hypotheses, PBI-loaded PCVs were first prepared by adding PTA (100 μ L, 22 mM, pH 6.5) to the PBI-containing coacervates (500 μ L PDDA, 50 μ L PBI-TEG, 550 μ L PAA). As expected, the resulting PTA-based PCVs retained strong red fluorescence, indicating the absence of quenching (Figure S26). When Ru₄POM (100 μ L, 6 mM) was added in place of PTA, PCVs formed after 3-7 days. These vesicles closely resembled the previously described 100% Ru₄PCVs in terms of size and membrane organization, as confirmed by polarized light microscopy (Figure 4.8a). Notably, the PBI-associated fluorescence was no longer observed. To directly compare the fluorescence behavior, a mixed population of PBI-loaded PTA PCVs and PBI-loaded Ru₄PCVs was imaged under identical microscopy conditions. A clear contrast in fluorescence was observed, with only the PTA-based PCVs exhibiting red emission (Figure 4.8b). To further assess whether fluorescence quenching was specifically associated with QS assembly, solid-state UV-vis spectroscopy was employed. As reported by Gobbato *et al.*,²⁷ QS formation can be monitored through characteristic changes in the PBI absorption spectrum: upon coordination to Ru₄POM, the PBI band at 542 nm decreases in intensity, while a new peak emerges at 562 nm. Consistent with this behavior, PBI-loaded PCVs formed using PTA showed no spectral changes, whereas those formed using Ru₄POM exhibited the appearance of a distinct shoulder at 562 nm (Figure 4.8c,d). This spectral signature strongly supports the formation of QS assemblies within the PCVs. These findings were further corroborated by hyperspectral microscopy analysis of the PCV membranes, which revealed spectral features consistent with QS formation exclusively in the Ru₄POM-containing vesicles (Figure S27).

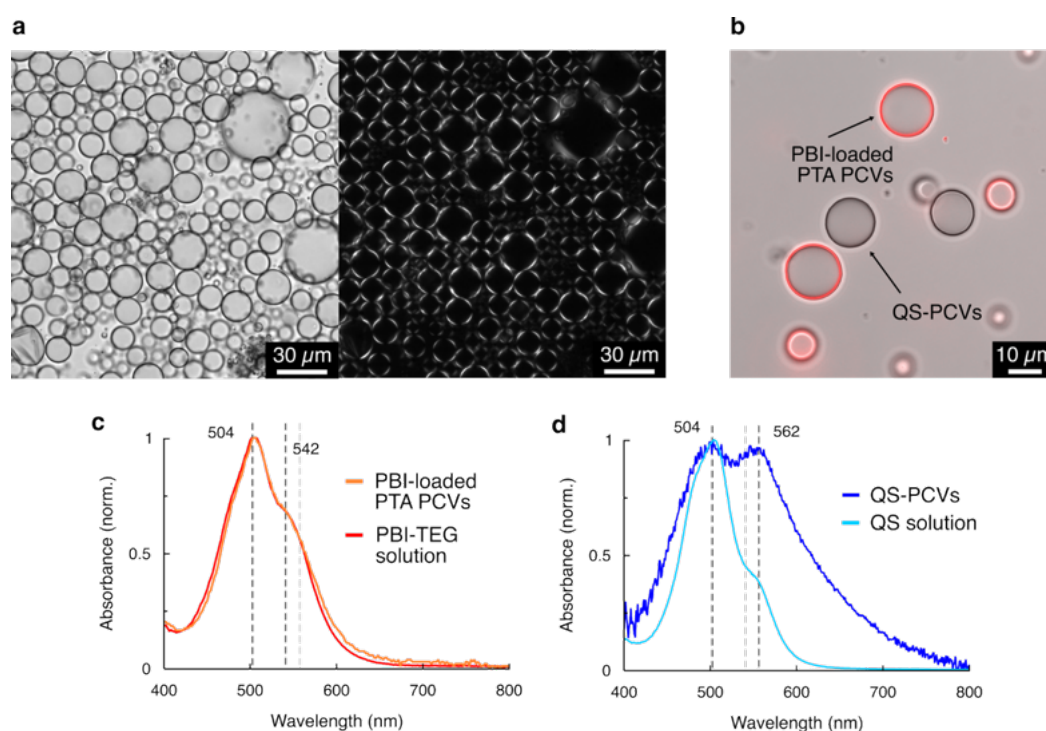


Figure 4.8 **a**, Brightfield (left) and cross-polarized (right) optical microscopy images of a population of QS-PCVs. **b**, Merged fluorescence and brightfield microscopy image of a mixed population of PBI-loaded PTA PCVs and QS-PCVs. Fluorescence is retained in PTA PCVs, whereas QS-PCVs show complete quenching. **c**, UV-vis spectra of solids samples of PBI-TEG solution (red plot) compared to the one of PBI-loaded PTA PCVs (orange plot). **d**, UV-vis spectra of solids samples of QS dispersed in water (light-blue plot) compared to the one of QS-PCVs (blue plot). The characteristic absorption features at 542 nm and 562 nm, associated with PBI and QS assemblies, respectively, are retained upon incorporation into the PCVs.

Collectively, these results indicate that the coacervate vesicle membrane provides a suitable environment for the *in situ* assembly of antenna-catalyst architectures.

4.5 CONCLUSIONS AND PERSPECTIVES

In this chapter, coacervate vesicles were developed as photocatalytic protocells for light-induced water oxidation, with a progressive increase in structural and functional integration. By replacing non-catalytic phosphotungstate species with the catalytic Ru₄POM, a new generation of vesicles was obtained in which the

polyoxometalate acts simultaneously as a structural component and as an active catalyst. The resulting 100% Ru₄PCVs exhibited well-defined vesicular morphology, organized membranes, and sufficient robustness to allow lyophilization and rehydration without loss of structural integrity. Photocatalytic experiments demonstrated that 100% Ru₄PCVs were active toward light-induced water oxidation in the presence of a soluble photosensitizer and sacrificial electron acceptor, representing a step toward more efficient and scalable protocellular architectures.

Building on this model, QS-PCVs were successfully constructed by integrating a self-standing antenna-catalyst assembly within the vesicle membrane. Spectroscopic and microscopic analyses provided evidence for the formation of quantasome architectures inside the PCVs, resulting in fluorescence quenching and characteristic absorption features. At present, these QS-PCVs are under investigation as fully integrated photocatalytic protocells capable of photoinduced water oxidation without an externally supplied photosensitizer. In parallel, the study of other quantasome nanosystems with different photosensitizers units is in progress, to expand the absorbance spectrum of the plankton-like protocells to new wavelengths (Figure S28).

Overall, the results presented in this chapter establish coacervate vesicles as a modular, non-equilibrium platform in which the integration of functional molecular components enables the emergence of increasingly complex coacervate-based systems with new photocatalytic functionalities.

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4.7 APPENDICES

4.7.1 Materials and methods

Milli-Q water was obtained from a Millipore Milli-Q Plus 185 apparatus and presented a resistivity of 18.2 MΩcm. All reagents were used as received unless otherwise stated. Poly(acrylic acid sodium salt) (PAA; 2.1 kDa), poly(diallyldimethylammonium chloride) solution (< 100 kDa; PDDA), sodium phosphotungstate hydrate ($\geq 99.9\%$) (PTA), adenosine 5'-triphosphate disodium salt hydrate (ATP), tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate ($\text{Ru}(\text{bpy})_3\text{Cl}_2$), sodium persulfate, sodium fluorosilicate (Na_2SiF_6), and sodium bicarbonate (NaHCO_3) were purchased from Sigma-Aldrich. Ru_4POM , $\text{PBI-TEG}_{\text{lock}}$ and $\text{PBI-TEG}_{\text{unlock}}$ have been synthesized by Prof. Bonchio's research group and used as received.

Optical and fluorescence microscopy was performed on an Axio Observer 7 (Zeiss) microscope at 20 $\times$ and 40 $\times$ magnification. The microscope was equipped with an Orca Flash 4.0 V3 CMOS camera (Hamamatsu) and a motorized XYZ sample stage. The brightfield images were obtained using a transmitted light LED lamp. Images were analyzed using the latest version of ImageJ software.

Confocal laser scanning microscopy images were obtained on a FV3000 (Olympus) confocal laser scanning microscope. The system was equipped with 4 excitation laser lines: 405, 488, 561, and 640 nm (Coherent), a galvanometric scanning head, and 4 spectral detectors, allowing for simultaneous imaging of up to 4 fluorescence channels. Brightfield images were acquired using a scanning laser line and a transmitted light detector. The inverted microscope body (Olympus IX 83) included a motorized automated sample stage for multi-area tiled imaging. The following objectives were used: UPLXAPO 20 $\times$ /0.8 NA (Olympus), and an oil objective UPLXAPO 60 $\times$ /1.42 NA (Olympus). Images were taken using excitation wavelength of 561 nm (RITC, for PBI samples) and emission wavelengths of 575-650 nm. Images were analyzed using the latest version of ImageJ software.

UV-vis spectroscopy was conducted using a Cary 5000 UV-Vis-NIR spectrophotometer, in the wavelength range of 175–900 nm. The instrument was equipped with tungsten-halogen lamps for the visible range and deuterium arc lamps for the UV range. To perform UV-vis analysis on solid samples we used an internal Diffuse Reflectance Accessory, the Cary DRA-2500 (PMT/PbS version).

A Seven Compact digital pH-meter by Mettler Toledo was used for pH determinations. The pH electrode was an InLab Semi-Micro glass electrode. The pH meter was calibrated before each set of measurements using standard buffer solutions at pH 4.01, 7.00, and 10.01, ensuring accurate readings across the relevant pH range.

Molecular oxygen concentration was measured using an OceanOptics NeoFox and FOSPOR R probe with a silicon overcoat. In-solution measurements involved a two-point calibration: maximum oxygen ($250 \mu\text{mol L}^{-1}$) was determined using a $\text{Na}_2\text{SiF}_6/\text{NaHCO}_3$ buffer solution, and minimum oxygen ($0 \mu\text{mol L}^{-1}$) was measured after purging with nitrogen for 15 min. Data was recorded using NeoFox Viewer version 2.40, with experiments conducted in reactors of 1 cm diameter.

4.7.2 Experimental

A1 – 100% Ru₄PCVs preparation. In a glass vial containing 500 μ L of a PDDA solution (MilliQ water, 10 mM, pH 6.5), 500 μ L of a PAA solution (MilliQ water, 20 mM, pH 6.5) were quickly added, and the mixture was stirred for 30 s at 1700 rpm to produce a turbid suspension of coacervate microdroplets. Subsequently, 100 μ L of an aqueous solution of 6 mM Ru₄POM was quickly injected into the vial to start the structural reconfiguration of the microdroplets. The PCV dispersion was stirred for another 30 s and then, after removing the magnetic stirrer, was allowed to rest for 7 days. After the PCVs formed, the dispersion was transferred into an Eppendorf tube and left to sediment for 30 min. The PCVs were then washed by removing the clear supernatant and redispersion in 850 μ L of MilliQ water. The washing procedure was repeated three times to give a batch of PCVs dispersed in 500 μ L of MilliQ water.

A2 – PBI-loaded PTA PCVs preparation. In a glass vial containing 500 μ L of a PDDA solution (MilliQ water, 10 mM, pH 6.5) and 50 μ L of PBI-TEG solution (MilliQ water, 5 mM), 550 μ L of a PAA solution (MilliQ water, 20 mM, pH 6.5) were quickly added, and the mixture was stirred for 30 s at 1700 rpm to produce a light-red turbid suspension of PBI-loaded coacervate microdroplets. Subsequently, 100 μ L of an aqueous solution of 22 mM PTA was quickly injected into the vial, resulting in the instantaneous generation of PCVs. The PCV dispersion was stirred for another 30 s and then, after removing the magnetic stirrer, was allowed to rest for a few minutes. After the PCVs formed, the dispersion was transferred into an Eppendorf tube and left to sediment for 30 min. The PCVs were then washed by removing the clear supernatant and redispersion in 850 μ L of MilliQ water. The washing procedure was repeated three times to give a batch of PCVs dispersed in 500 μ L of MilliQ water.

A3 – QS-PCVs preparation. In a glass vial containing 500 μ L of a PDDA solution (MilliQ water, 10 mM, pH 6.5) and 50 μ L of PBI-TEG_{unlock} solution (MilliQ water, 5 mM), 550 μ L of a PAA solution (MilliQ water, 20 mM, pH 6.5) were quickly added, and the mixture was stirred for 30 s at 1700 rpm to produce a light-red turbid suspension of PBI-loaded coacervate microdroplets. Subsequently, 100 μ L of an

aqueous solution of 6 mM Ru₄POM was quickly injected into the vial, resulting in the instantaneous generation of PCVs. The PCV dispersion was stirred for another 30 s and then, after removing the magnetic stirrer, was allowed to rest for 7 days. After the PCVs formed, the dispersion was transferred into an Eppendorf tube and left to sediment for 30 min. The PCVs were then washed by removing the clear supernatant and redispersion in 850 μ L of MilliQ water. The washing procedure was repeated three times to give a batch of PCVs dispersed in 500 μ L of MilliQ water.

A4 – Photocatalytic water oxidation experiments. 40 μ L of a solution of Ru(bpy)₃Cl₂ (MilliQ water, 50 mM) and the required amount of Ru₄PCVs were suspended in 1.85 mL of Na₂SiF₆/NaHCO₃ buffer (3.8 mM in Na₂SiF₆ and 6.2 mM in NaHCO₃, pH 5.6) and stirred at 1,200 rpm in a reactor vessel. The suspension was kept in the dark and purged with nitrogen for 15 min. Subsequently, 100 μ L of a solution of Na₂S₂O₈ (MilliQ water, 100 mM) was added to the reactor, the reactor was sealed, and the dioxygen level was monitored for 5 min to acquire a baseline measurement. After 5 min of equilibration time, a white light (white LED lamp 442 μ W, 2.78 mW cm⁻²) was turned on to trigger photocatalysis, and the dioxygen level was monitored for at least 1 h (solution measurements) at room temperature. The same procedure was used to carry out photocatalytic water oxidation experiments using Ru₄POM, with the exception that 10 μ L of a freshly prepared Ru₄POM stock solution (5 μ M) in MilliQ water was used instead of the Ru₄PCVs. The initial rate of dioxygen production was obtained from the slope of the linear part of the dioxygen curve obtained from solution measurements in the first two minutes.

A5 – Microscopy imaging. Where not specified, microscopy images of PCVs were obtained by injecting 50 μ L of protocells dispersion in MilliQ water in a glass channel slide of a Petri dish. To take over time images of PCVs evolving over several days, a microscopy channel slide loaded with the sample was sealed with hydrophobic grease and Parafilm®.

A6 – UV-vis spectroscopy on PCVs. To study solid samples of PCVs, we dispersed a batch in 200 μ L of MilliQ water in an Eppendorf tube. A 100 μ L aliquot of this

dispersion was drop-cast onto a thin glass substrate and allowed to dry under controlled conditions. After solvent evaporation, the resulting thin film of dried PCVs was directly mounted onto the DRA-2500 integrating sphere for optical measurements.

UV-vis data were collected as absolute reflectance (%R) and converted to absorbance using the Kubelka-Munk function (Equation S1):

$$Abs = \frac{(100 - \%R)^2}{2 \times \%R} \quad \text{Equation S1}$$

Because concentration values cannot be defined for solid samples, spectral normalization was performed by subtracting the spectrum of an appropriate reference sample lacking the PBI-TEG chromophore, followed by normalization. Prior to subtraction, spectra were aligned by setting the baseline to zero in a wavelength region outside the PBI absorption range (800 nm).

Specifically, for PBI-loaded PTA PCVs, the spectrum of unloaded PTA PCVs was subtracted. For QS-PCVs, the spectrum of 100% Ru4PCVs was used as the reference.

4.7.3 Supplementary figures

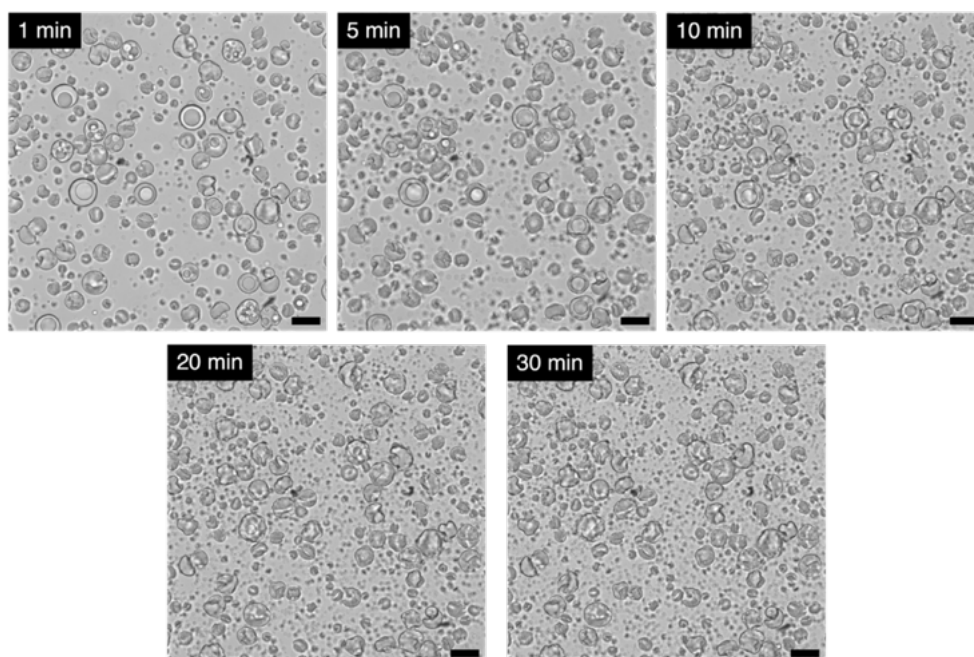


Figure S22 Optical microscopy images of a population of PDDA/ATP coacervate microdroplets after the addition of a Ru₄POM solution, over time. The spherical vesicles collapse after few minutes and lose the internal aqueous lumen. Scale bars = 30 μ m.

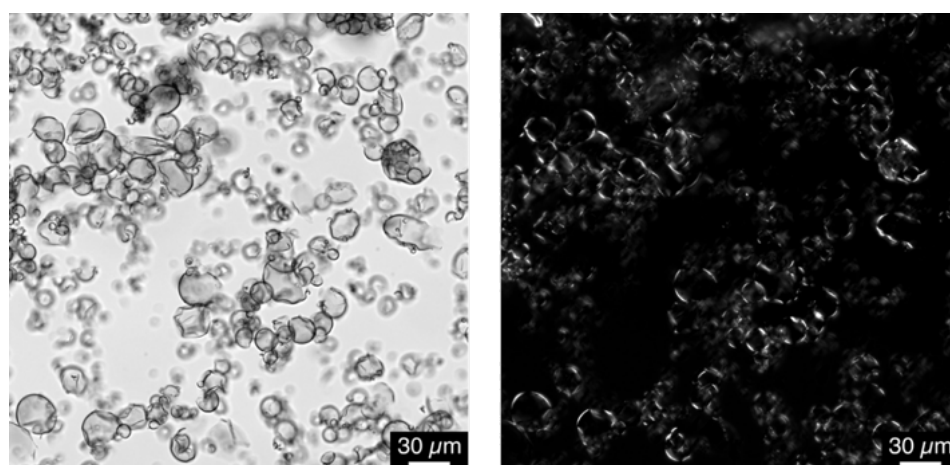


Figure S23 Brightfield and cross-polarized microscopy images of a population of 100% Ru₄PCVs after washing, lyophilization, and re-dispersion in Milli-Q water. The vesicles retain their membraned structure and characteristic birefringence pattern.

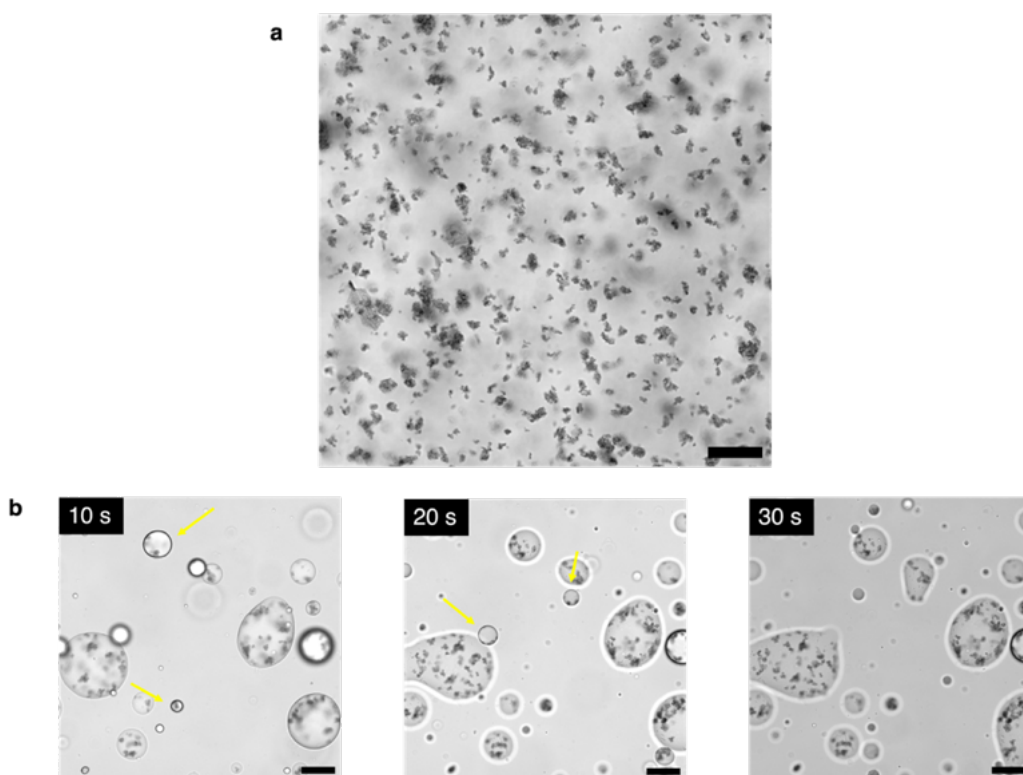


Figure S24 **a**, Brightfield microscopy image of quantasome (PBI-TEG and Ru₄POM, 5:1 molar ratio) aggregates dispersed in water. **b**, Brightfield microscopy images of quantasome aggregates that get sequestered within PDDA/PAA coacervate microdroplets, over time. The coacervates are not stable and collapse on the glass surface. Yellow arrows indicate single droplets depositing on the glass. Scale bars are 30 μm .

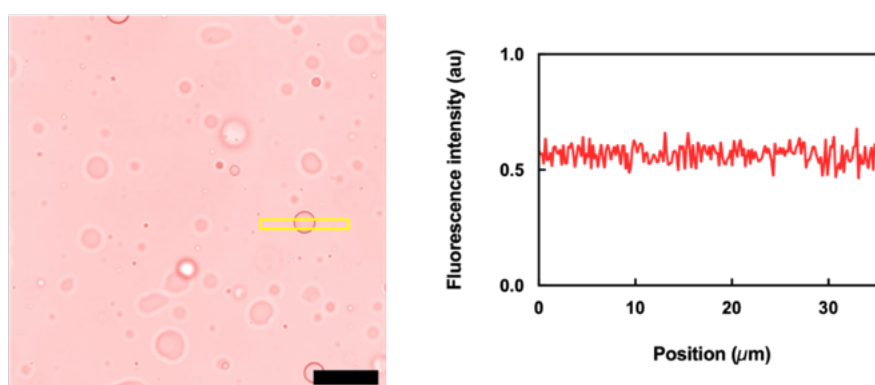


Figure S25 Merged brightfield and fluorescence representative confocal microscopy image of PBI-TEG-loaded PDDA/PAA coacervate microdroplets. Normalized fluorescence intensity line profile for the yellow area drawn on the microscopy image is reported in the graphs on the right. PBI-TEG seems to partition equally inside and outside the PDDA/PAA coacervate microdroplets. Scale bar is 30 μm .

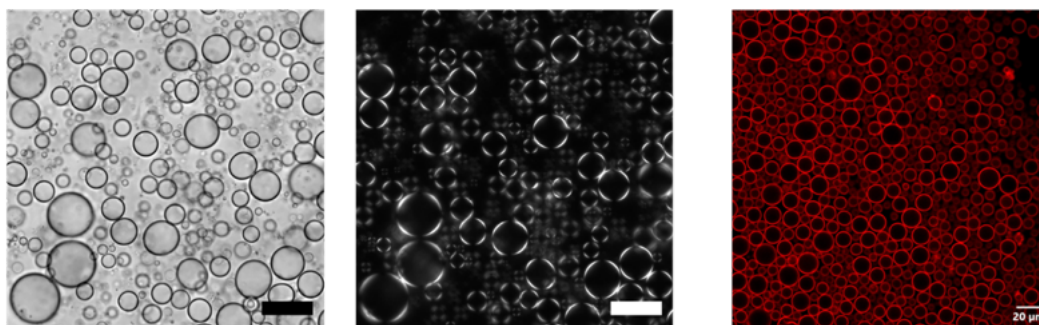


Figure S26 Brightfield (left), cross-polarized (middle), and confocal fluorescence microscopy images (right) of a population of PBI-loaded PTA PCVs. Where not reported, scale bars = 30 μm .

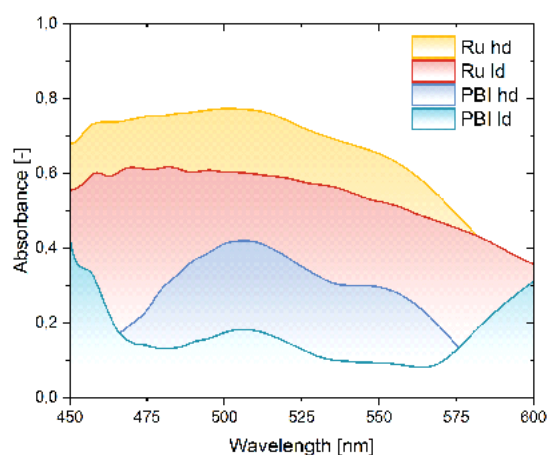


Figure S27 Hyperspectral microscopy absorbance spectra of PBI-loaded PTA PCVs (blue plot) and QS-PCVs (yellow plot) samples. Green and red plots refer to areas of the samples with less density of PCVs. It is possible to see how, passing from the PBI sample alone to the QS one, the peak at 500 nm keeps its spectral position while broadening, and the peak at 550 nm has a weak redshift and broadening. This qualitative observation is in agreement with previously conducted absorption studies on the QS system.²⁸ Measurements and data analysis have been performed by Dr. Mattia Russo, POLIMI, Milan, IT.

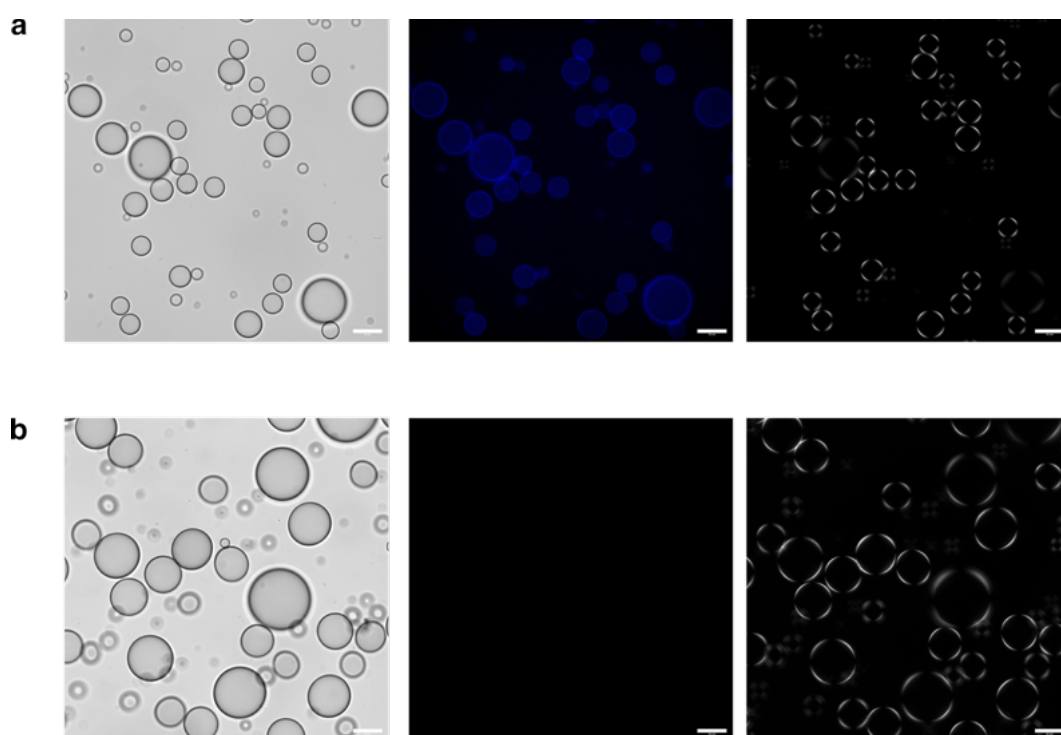


Figure S28 Replacing the photosensitizer with functionalized naphthalene diimide (NDIBlue) did not alter the outcome. Brightfield (left), fluorescence (middle), and cross-polarized (right) microscopy images of (a) NDIBlue-loaded PTA PCVs and (b) NDIBlue-loaded Ru₄PCVs. In both cases, PCVs readily form; however, a complete quenching of blue fluorescence is observed for the Ru₄PCVs, indicating quantasome formation. Scale bars = 30 μm

5

CONCLUSIONS AND OUTLOOK

5.1 ON THE FORMATION OF COACERVATE SYSTEMS

Complex coacervates are supramolecular, out-of-equilibrium systems that can closely mimic key dynamic features of living materials. They form via liquid-liquid phase separation of oppositely charged polyelectrolytes in aqueous solution. By selecting appropriate polyelectrolyte pairs and solution conditions, coacervate microdroplets can be generated as microcompartmentalized units that have been widely investigated as protocellular models due to their ability to concentrate reactants, enhance enzymatic reactions, and support dynamic chemical processes.

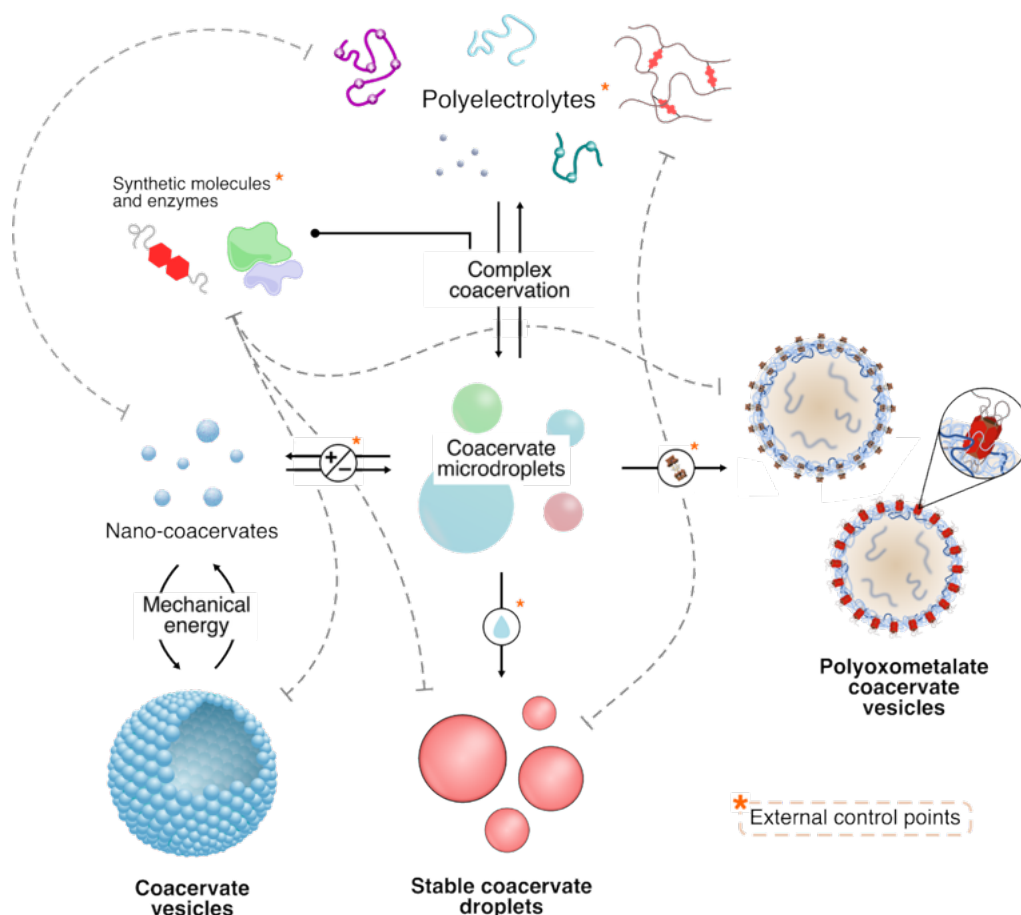


Figure 5. 1 Conceptual map illustrating the pathways for the synthesis and functionalization of coacervate-based protocells presented in this thesis. Solid arrows indicate formation pathways from

molecular building blocks to structured coacervate assemblies. Dashed lines denote conceptual relationships between different components of the diagram, including given features and functionalities. Orange stars highlight external intervention points where the final protocellular outcome can be modulated by changing molecular components, triggering structural transformations, or introducing specific functionalities.

Despite these promising characteristics, coacervate microdroplets are intrinsically unstable and tend to rapidly coalesce into a single dense phase, which limits their suitability for performing complex and sustained protocellular functions.

In this thesis, we demonstrate that by carefully tuning key parameters of the formation process, coacervate systems can be driven into novel and more robust architectures suitable for functional applications. Specifically, we show that mechanical energy can be exploited to induce the reconfiguration of stable nano-coacervates, formed outside the stoichiometric coacervation ratio, into organized and stable coacervate vesicles. This mechanism was subsequently extended to different polyelectrolyte combinations, highlighting its generality and versatility. We further investigated a simple yet effective strategy to generate highly stable coacervate droplets directly from dense complex phases through controlled dilution and mechanical agitation. These droplets, prepared from functional synthetic polymers, exhibited enhanced stability and molecular sequestration capabilities. Finally, within the framework of polyoxometalate-based coacervate vesicles, we showed that selective control over vesicle composition can be achieved by rationally adjusting the polyelectrolyte components. Importantly, the incorporation of functional molecules into the coacervate phase enabled the formation of complex supramolecular assemblies during the droplet-to-vesicle transition, yielding architectures that may display living organoid-like features.

Together, these results highlight coacervates as adaptive soft-matter systems whose structure can be dynamically tuned across equilibrium and non-equilibrium regimes.

5.2 EMERGENT FUNCTIONALITIES AND APPLICATIONS

Beyond structural control, the integration of functional (macro)molecules enables coacervate compartments to perform increasingly complex chemical tasks. In this thesis, mechanically driven dissipative self-assembly of coacervate vesicles was combined with the intrinsic ability of coacervates to sequester enzymes, yielding catalytic protocells that transiently evolve over time. By preparing two distinct protocell populations containing either glucose oxidase or horseradish peroxidase, and by exploiting their reversible dissipative behavior, it was possible to generate an evolved protocell population in which both enzymes coexisted while remaining spatially organized. This reconfiguration significantly enhanced the efficiency of the enzymatic cascade, demonstrating how mechanical energy can drive the progression from simple, separated units toward more integrated and functionally advanced protocellular systems.

In a complementary approach, the urea-urease enzymatic reaction network was successfully embedded within coacervate microdroplets to generate nonlinear chemical dynamics. These dynamics were transduced into optical signals through photoinduced electron transfer (PET) polymers, effectively coupling chemical work to real-time information readout. Importantly, the intrinsic properties of the constituent polyelectrolytes were retained in the final droplets, resulting in dynamic systems capable of translating chemical information into diverse outputs.

Finally, coacervate vesicles functionalized with catalytic polyoxometalates illustrate how structural organization and catalysis can be integrated within a single protocellular entity. The development of fully catalytic and antenna-integrated photocatalytic vesicles further demonstrates the ability of coacervate systems to support light-driven reactions, including water oxidation. Collectively, these examples

highlight the potential of coacervates as programmable microreactors in which structure, dynamics, and function are intrinsically coupled.

5.3 OUTLOOK AND FUTURE PERSPECTIVES

Looking forward, the systems presented in this thesis open multiple avenues for the development of increasingly autonomous and life-like protocells. The combination of non-equilibrium assembly, compartmentalized catalysis, and signal transduction provides a robust foundation for constructing systems capable of feedback, communication, and adaptive behavior. Future research may focus on extending the concepts introduced here to a broader range of functional polyelectrolyte mixtures and catalytic machineries, further expanding the accessible chemical and physical properties of coacervate-based systems.

The concept of mechanically driven dissipative self-assembly of coacervate vesicles may be further exploited to develop self-standing carrier-drug delivery platforms, explore plausible origin-of-life scenarios, and design advanced protocellular models with emergent functionalities. Additional efforts could aim to couple multiple reaction networks within single compartments, implement internal regulatory mechanisms, or enable protocell-protocell interactions to achieve collective and cooperative dynamics. From a materials perspective, improvements in robustness, scalability, and long-term stability will be essential for translating coacervate protocells into functional soft materials. More broadly, this work underscores how embracing non-equilibrium principles and molecular integration can promote the emergence of complexity in synthetic systems, bridging soft matter chemistry, supramolecular design, and synthetic biology.



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