

Biogenic Condensation Pathway of “All-Nucleotide” Hydrogels: Towards Biocatalytic Cascades

M.Sc. Thesis

By

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**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY INDORE**

May 2026

Biogenic Condensation Pathway of “All-Nucleotide” Hydrogels: Towards Biocatalytic Cascades

A THESIS

*Submitted in partial fulfilment of the
requirements for the award of the degree*

of

Master of Science

by

Divyanshi Deshwal



**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY INDORE**

May 2026



INDIAN INSTITUTE OF TECHNOLOGY INDORE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled ***“Biogenic condensation Pathway of “All Nucleotides” Hydrogels: Towards Biocatalytic Cascades”*** in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF CHEMISTRY, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 21, 2025, to May 8, 2026, under the supervision of Dr. Tridib Kumar Sarma, Discipline of Chemistry, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Signature of the student with date
(DIVYANSHI DESHWAL)

This is to certify that the above statement made by the candidate is accurate, complete, and correct to the best of my knowledge and belief.

Signature of the supervisor with date
(Dr. Tridib Kumar Sarma)

Divyanshi Deshwali has successfully given her oral examination held on 08/05/2026.

Signature of the supervisor
Date: 18/05/2026

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, Dr. Tridib Kumar Sarma, for his constant guidance, encouragement, and invaluable support throughout the course of this project. Working under his supervision has been an enriching and inspiring experience. His insightful suggestions, constructive criticism, and continuous motivation helped me overcome various challenges and gain a deeper understanding of the fundamental principles related to my research. I am truly grateful for his mentorship and for encouraging my future academic pursuits.

I would also like to extend my heartfelt thanks to the faculty members of the Department of Chemistry, especially Prof. Anjan Chakraborty and Dr. Pravarthana Dhanapal, my Course Coordinators, for their valuable guidance, support, and assistance throughout different aspects of this work.

I sincerely acknowledge all the teachers of my department and staff members for their technical assistance and cooperation. I am also thankful to the Sophisticated Instrumentation Centre and Indian Institute of Technology Indore for providing the necessary research facilities and infrastructure to successfully carry out this work.

I express my profound gratitude to my mentor, Mr. Suryakamal Sarma, whose continuous support, guidance, and assistance in every aspect of this project were truly invaluable. His encouragement and willingness to help at every stage greatly contributed to the successful completion of this work.

I am deeply thankful to my lab mates - Dr. Vidhi, Mr. Aditya, Mr. Tarun, Mr. Ravindra, Ms. Anjali, Ms. Yoshita, and Ms. Anamika for their constant help, encouragement, and companionship in both academic and non-academic matters. I also extend my sincere thanks

to the students of Prof. Anjan Chakraborty's laboratory, as well as all my friends and batchmates, for their support and memorable moments throughout this journey.

Finally, I would like to express my deepest love and gratitude to my beloved parents, Mr. Bhupendra Kumar and Mrs. Surekha Devi for their unconditional love, endless sacrifices, unwavering faith, and constant encouragement, which have always been my greatest source of strength. I also convey my heartfelt regards to my dear brothers, Mr. Saurabh Deshwal and Mr. Deepanshu Deshwal, for their love and support.

A special note of gratitude goes to Mr. Ashish Salaklan for always supporting, motivating, and believing in me during difficult times. I am equally thankful to my dear friends Anahita, Anamika, Meet, and Narottam for their constant encouragement, understanding, and cherished friendship throughout this journey.

Divyanshi Deshwal

(M.Sc. Student)

Dedicated to my father....

ABSTRACT

Biomolecular condensation offers a powerful strategy to create confined microenvironments for regulating chemical transformations; however, translating this concept into minimal small-molecule catalytic systems remains largely unexplored. Herein, we report an “all-nucleotide” platform that spontaneously condenses into hierarchically organized G-quadruplex (G4)-based supramolecular networks and hydrogels with intrinsic biocatalytic activity. Co-assembly of guanosine 5'-monophosphate (GMP) with adenosine phosphate derivatives reveals that phosphate multiplicity and intrinsic acidity cooperatively govern hierarchical self-assembly. While adenosine monophosphate (AMP) exhibits limited organization, adenosine di- and triphosphates (ADP and ATP) drive a concentration-dependent transition from liquid-liquid phase-separated droplets to fibrillar networks, with GMP serving as the primary structural building block. Mechanistic investigations demonstrate that multivalent phosphate interactions and local pH modulation regulate charge screening and proton redistribution, thereby promoting G-quartet stacking. Notably, ADP affords superior structural organization due to optimal alignment within favourable pH window. At higher concentrations, both ADP-GMP and ATP-GMP systems form stable, self-supporting hydrogels through higher-order assembly. These hydrogels act as efficient, metal-free peroxidase mimics, exhibiting enhanced substrate affinity compared to native HRP, enzyme-like kinetics, and a hydroxyl radical-mediated catalytic pathway. Furthermore, they function as dual-purpose systems, serving as both catalytic scaffolds and biomimetic microenvironments, thereby enabling enzyme encapsulation and multi-step cascade reactions with improved kinetics and sensitive detection. This work establishes a minimalistic strategy that integrates biomolecular condensation with catalysis, providing a versatile platform for artificial microreactors, biosensing, and protocell-inspired materials.

TABLE OF CONTENTS

List of Figures	iii-viii
List of Schemes	ix
Abbreviation	x
Acronyms	xi
Chapter 1: Introduction	1-5
Chapter 2: Literature Survey	6-14
Chapter 3: Motivation	15-16
Chapter 4: Objective	17
Chapter 5: Experimental Details	18
5.1. Materials	18
5.2. Methods	18-19
5.3. Synthetic Methods	20-23
Chapter 6: Results & Discussion	24
6.1. Condensation of Nucleotides	24-26
6.2. Kinetic Morphological Studies	26-29
6.3. UV-vis Spectroscopy	29-30
6.4. Circular Dichroism Spectroscopy	30-31
6.5. ATR-FTIR Spectroscopy	31-32
6.6. ThT Assay	33-36
6.7. pH Titration	36-37
6.8. Zeta Potential Analysis	37-38
6.9. Mechanism	38-40

6.10. ADG and ATG extensive condensation leading to hydrogelation	40-42
6.11. Rheological Analysis	42-43
6.12. Intrinsic Peroxidase Mimicking Activity	44-50
6.13. Enabling Biocatalytic Cascade with “All-Nucleotide” Hydrogel	50-54
Chapter 7: Conclusion	55-56
Chapter 8: Future Scope	57
References	58-62

List of Figures

Figure 1: Supramolecular hydrogels are made from basic biological building blocks like amino acids, peptides, nucleic acid motifs, and saccharides that self-assemble into a 3D network through non-covalent interactions.

Figure 2: (a) Chemical structures of purine and pyrimidine bases, with hydrogen-bond donor sites highlighted in red and acceptor sites in green. (b) Representation of Watson-Crick and Hoogsteen hydrogen bonding patterns. (c) Molecular structure of the A:A base pair illustrating electrostatic interactions. (d) Molecular structure of the hemi-protonated C:C⁺ base pair and (e) the corresponding molecular structure and schematic representation of a G-quadruplex.

Figure 3: (a) DNA macromolecular assembled into a G4-condensate that leads to the formation of a helical fibrillar network, and (b) Transformation of DNA-GMP condensate to DNA-GMP hydrogel at higher order condensation.

Figure 4: Acidic amino acid mediated phase separation promotes 5'-GMP self-assembly. (a) Chemical structures of aspartic acid and 5'-GMP, along with schematic representations illustrating G-quadruplex (G4) stacking and the ensuing supramolecular fibrillation. (b) Confocal microscopy images (DIC and green fluorescence channels) showing AA-GMP condensates during the initial condensation stage ([GMP] = 10 μ M); scale bar: $\sim$ 500 nm. (c) Morphological evolution from homogeneous condensates to phase-separated short fibrils and ultimately to nanofibrous networks with increasing 5'-GMP concentration; scale bar: $\sim$ 5 μ m.

Figure 5: (A) Time-lapse microscopic images of a freshly mixed protein-ATP sample observed in the brightfield mode (left column) and ThT emission-enhancing fluorescence mode (right column). (B) AFM amplitude images capturing the intermediate LLPS-to-fibril structural

transition. Images 1, 2, and 3 are the magnified sections of the image shown in the top left panel. Overlaid are cross-sections of selected specimen, as indicated by the likewise colored cross-lines, based on the corresponding height images.

Figure 6: Dynamics of a protein–ATP system under enzymatic perturbation, showing an unusual reverse transition from fibrils to droplets.

Figure 7. Schematic illustration of (a) two-enzyme (GOx/ZIF-8A and HRP/ZIF-8A) and (b) three-enzyme (b-gal/ZIF-8A, GOx/ZIF-8A and HRP/ZIF-8A) cascades “switch on” driven by complementary coiled-coil forming peptide (JR2EK and JR2KC) induced MOF superstructures and then “switch off” by chemically disrupting the coiled-coil superstructure.

Figure 8. Incompatible cascade reactions in pAAm@ZIF-8. Schematic illustration of the co-encapsulation (a) and spatial arrangement (b) of CAT with GOx/HRP cascade in pAAm@ZIF-8.

Figure 9. Tyndall effect for the visual confirmation of AMG, ADG, and ATG condensate formation, showing the laser beam passing through the condensate vial.

Figure 10. FE-SEM micrographs of the condensates (a) AMG, (b) ADG and (c) ATG systems. (sample concentration $\sim 100 \mu\text{M}$); *scale bar: 1 μm .*

Figure 11. Kinetic morphological evolution of the ADG system with varying GMP concentrations, illustrating the transition from droplet-like condensates to fibrillar networks. FESEM (a-d) and CLSM (e-i) micrographs show: (a,e) $10 \mu\text{M}$ GMP, formation of droplet-like assemblies; (f) $30 \mu\text{M}$ GMP, aggregation of droplets; (b,g) $50 \mu\text{M}$ GMP, emergence of protofibrils; (c,h) $70 \mu\text{M}$ GMP, development of fractal-like structures; and (d,i) $100 \mu\text{M}$ GMP, formation of an interconnected fibrillar network. FESEM: (*Scale bar $\sim 1 \mu\text{m}$*) and CLSM: (*Scale bar $\sim 5 \mu\text{m}$*).

Figure 12. Kinetic morphological evolution of the ATG system with varying GMP concentrations, illustrating the transition from droplet-like condensates to fibrillar networks. FESEM (a-d) and CLSM (e-i) micrographs show: (a,e) 10 μM GMP, formation of droplet-like assemblies; (f) 30 μM GMP, aggregation of droplets; (b,g) 50 μM GMP, emergence of protofibrils; (c,h) 70 μM GMP, development of fractal-like structures; and (d,i) 100 μM GMP, formation of an interconnected fibrillar network. FESEM: (Scale bar $\sim 1 \mu\text{m}$) and CLSM: (Scale bar $\sim 5 \mu\text{m}$).

Figure 13: UV-vis spectroscopic analysis and Job's plot for (a,b) AMP-GMP, (c,d) ADP-GMP, and (e,f) ATP-GMP systems. The GMP concentration was systematically varied from 10 to 100 μM , while the concentrations of AMP, ADP, and ATP were fixed at 100 μM .

Figure 14: Circular dichroism (CD) spectra of nucleotide components and their condensates: (a) AMP, GMP, and AMP-GMP condensate (AMG); (b) ADP, GMP, and ADP-GMP condensate (ADG); (c) ATP, GMP, and ATP-GMP condensate (ATG). Concentration-dependent CD spectra of d) ADG and (e) ATG recorded by varying GMP from 10 to 100 μM , while ADP and ATP concentrations were fixed at 100 μM .

Figure 15: ATR-FTIR spectra: (a) AMP, GMP, AMP-GMP condensate (AMG); (b) ADP, GMP, ADP-GMP condensate (ADG); (c) ATP, GMP, ATP-GMP condensate (ATG) by varying GMP concentration from 10-100 mM, keeping AMP, ADP and ATP concentrations maintained at 100 mM.

Figure 16. (a) Schematic representation of ThT binding within G-quadruplex channels. (b) Fluorescence excitation and emission spectra of Thioflavin T (ThT)-loaded ADG condensates, showing an excitation maximum at 350 nm and an emission maximum at $\sim 450 \text{ nm}$ ($\lambda_{\text{ex}} = 350 \text{ nm}$). (c) Comparison of fluorescence emission spectra of pristine ThT and ThT-loaded condensates, demonstrating enhanced emission upon binding to fibrillar assemblies. (d) Relative fluorescence enhancement

(FL/FL₀) compared to free ThT, showing ThT incorporated in AMG, ADG, and ATG condensates at an optimal concentration of 100 μ M.

Figure 17. (a-c) Fluorescence responses of AMG, ADG, and ATG condensates, respectively, as a function of increasing GMP concentration. (d) Concentration-dependent fluorescence emission of ADG and ATG condensates with varying ADP and ATP concentrations, respectively, at a fixed GMP concentration (100 μ M). (e) Temperature-dependent fluorescence showing decreased intensity due to thermal disruption of G-quartet stacking, with ADG exhibiting greater stability than ATG.

Figure 18: pH titration of the condensates. (a) AMP, GMP, AMP-GMP condensate (AMG); (b) ADP, GMP, ADP-GMP condensate (ADG); (c) ATP, GMP, ATP-GMP condensate (ATG) by varying GMP concentration from 0.1-1 mM, keeping AMP, ADP and ATP concentrations maintained at 1 mM.

Figure 19: Zeta potential analysis of nucleotide condensates: (a) AMP-GMP condensate (AMG), (b) ADP-GMP condensate (ADG), and (c) ATP-GMP condensate (ATG), compared to their respective monomeric nucleotides AMP, ADP, ATP, and GMP, each at 1 mM concentration.

Figure 20. Proposed mechanism illustrating GMP-driven G-quadruplex formation and π - π stacking, assisted by multivalent phosphate interactions, resulting in hierarchical fibrillar assembly and hydrogel formation.

Figure 21. Rheological and mechanical characterization of ADG and ATG hydrogels. (a) Amplitude sweeps measurements at a constant angular frequency of 10 rad.s⁻¹, (b) Frequency sweep measurements at constant 1% strain. (c, d) GMP concentration-dependent rheological analysis indicating the minimum and optimal gelation concentrations for ADG and ATG systems.

Figure 22: Peroxidase-like activity of ADP-GMP hydrogel: (a) Schematic of the intrinsic peroxidase-like catalysis. (b) Controlled

kinetic parameter with respect to the native biomolecules ADP and GMP (inset showing the kinetic graphs). (c) Time-derived oxidation of TMB to ox-TMB. (d) Controlled experiments for the different conditions of the peroxidase-like activity.

Figure 23: Michaelis-Menten-like enzymatic curves of the ADP-GMP hydrogel with respect to (a) TMB concentration (0.05–1.0 mM) and (b) H_2O_2 concentration (0.0–12 mM). Lineweaver-Burk plot of the ADP-GMP hydrogel with respect to the change in (c) TMB concentration and (d) H_2O_2 concentration.

Figure 24. (a) Specific activity curve when TMB is used as substrate. (b) Effect of temperature on the peroxidase activity. (c) Concentration-dependent enhancement in catalytic velocity with increasing GMP content in the ADG hydrogel, highlighting the critical role of G-quadruplex (G-quartet) structures in governing catalytic activity.

Figure 25: (a) Schematic reaction showing 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin-trapping agent. (b) Electron paramagnetic resonance (EPR) spectra obtained, showing a characteristic four-line signal corresponding to the $\text{DMPO}\cdot\text{OH}$ adduct in the presence of ADG and ATG systems.

Figure 26. Fluorescence spectra of $\cdot\text{OH}$ generated in the reaction system: (a) Schematic illustration of the conversion of terephthalic acid to fluorescent 2-hydroxyterephthalic acid upon reaction with hydroxyl radicals ($\cdot\text{OH}$). (b) in the presence of the catalyst or in the absence of the catalyst. (c) Time-dependent Fluorescence of terephthalic acid.

Figure 27. Schematic illustration of enzyme encapsulation (β -galactosidase and glucose oxidase) within the ADG hydrogel, highlighting its dual role as a confined catalytic milieu and intrinsic HRP-like scaffold.

Figure 28. (a) A two-enzyme cascade system in which GOx catalyzes the oxidation of glucose to produce H_2O_2 , which is subsequently utilized by the hydrogel to oxidize TMB into ox-TMB. (b) Photographic images

showing concentration-dependent colorimetric response for glucose for the 2-step. (c) Calibration curves illustrating the linear correlation between absorbance and glucose concentration at a fixed reaction time (30 min) and constant TMB concentration (1 mM) for the cascade systems, along with corresponding limits of detection (LOD).

Figure 29. (a) Three-enzyme cascade system involving β -Gal-mediated hydrolysis of lactose to glucose, followed by GOx-generated H_2O_2 and hydrogel-catalysed TMB oxidation. (b) Photographic images showing concentration-dependent colorimetric response for laccase for the 3-step. (c) Calibration curves illustrating the linear correlation between absorbance and lactose concentration at a fixed reaction time (30 min) and constant TMB concentration (1 mM) for the cascade systems, along with corresponding limits of detection (LOD).

List of Schemes

Scheme 1: Schematic representation of the condensation of GMP with AMP, ADP and ATP.

Scheme 2. Schematic representation of the transformation of a fibrillar condensate (AMG) to a homogeneous transparent hydrogel at higher order self-assembly (ADG, ATG). Diagram depicting gelation (✓) and non-gelation (X) as a function of concentration, highlighting the critical threshold required for hydrogel formation.

Abbreviation

Degree Celsius	°C
Gram	g
Hour	h
Milligram	mg
Millilitre	mL
Millimole	mmol
Micro Molar	μM
Micro Litre	μL
Unit	U

Acronyms

LLPS	Liquid-liquid phase separation
G4s	G-quadruplexes
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
ATPNa ₂	Adenosine Triphosphate disodium Salt
ADPNa ₂	Adenosine Diphosphate disodium Salt
AMPNa ₂	Adenosine Monophosphate disodium Salt
GMPNa ₂	Guanosine Monophosphate disodium Salt
UV-vis	Ultraviolet visible
CLSM	Confocal Laser Scanning Microscopy
HR-TEM	High Resolution Transmission Electron Microscopy
CD	Circular Dichroism
FESEM	Field Emission Scanning Electron Microscopy
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared
AS	Amplitude Sweep
FS	Frequency Sweep
HRP	Horseradish peroxidase
β-Gal	β-galactosidase
Gox	Glucose oxidase
DMPO	5,5-dimethyl-1-pyrroline N-oxide
EPR	Electron paramagnetic resonance
TA	Terephthalic acid

Chapter 1

Introduction

Spatiotemporal regulation in complex biological systems arises from the controlled compartmentalization within the cellular matrix.¹ While traditional organelles like the endoplasmic reticulum and Golgi apparatus are enclosed by lipid bilayer membranes, many other cellular compartments lack such boundaries.² Examples of these membrane-less organelles include nucleoli, heterochromatin, nuclear pore complexes, and membrane receptor clusters.^{3,4} These compartments form through a process known as liquid-liquid phase separation (LLPS), where one or more macromolecules separate into two coexisting liquid phases: a dense phase enriched with the molecules and a dilute phase depleted of them. In biological systems, this process is also called liquid-phase condensation, and the resulting dense phases are known as biomolecular condensates.^{5,6} LLPS typically occurs when macromolecules with complementary interactions, such as electrostatic attraction between oppositely charged polyelectrolytes are mixed in aqueous solutions under suitable conditions. The assemblies formed can exhibit a range of physical states, from dynamic liquids to viscous gels or solid-like aggregates.^{7,8} LLPS is often compared to complex coacervation, where droplet-like structures enriched in solutes appear under a light microscope, separated from a surrounding dilute phase.⁹ The term “biomolecular condensate” is now widely used to describe these dynamic assemblies, which may exist as reversible droplets, gels, or networks formed through multivalent interactions. Molecules with multiple binding sites can connect to form dynamic networks, reducing solubility and promoting phase separation. Overall, phase separation provides a unifying framework for understanding how biological macromolecules organize into membrane-less compartments.^{5,10,11}

Recent research has highlighted new strategies that could replace traditional stable assemblies by creating compartments resembling membrane-less organelles.^{12,13} These artificial condensates, often made

from nucleotides, show tunable properties such as polarity, molecular diffusion, and selective molecule recruitment in response to external stimuli.¹⁴ Most existing studies have focused on complexes formed by intrinsically disordered proteins with DNA or RNA through electrostatic interactions.¹⁵ However, nucleotides alone can also self-assemble into compartment-like systems. Among such systems, assemblies formed by two different nucleotides through non-electrostatic interactions, especially those generating G-quadruplex (G4) structures remain less explored. G4s are known to participate in essential cellular functions such as DNA replication, meiosis, and telomere maintenance.¹⁶

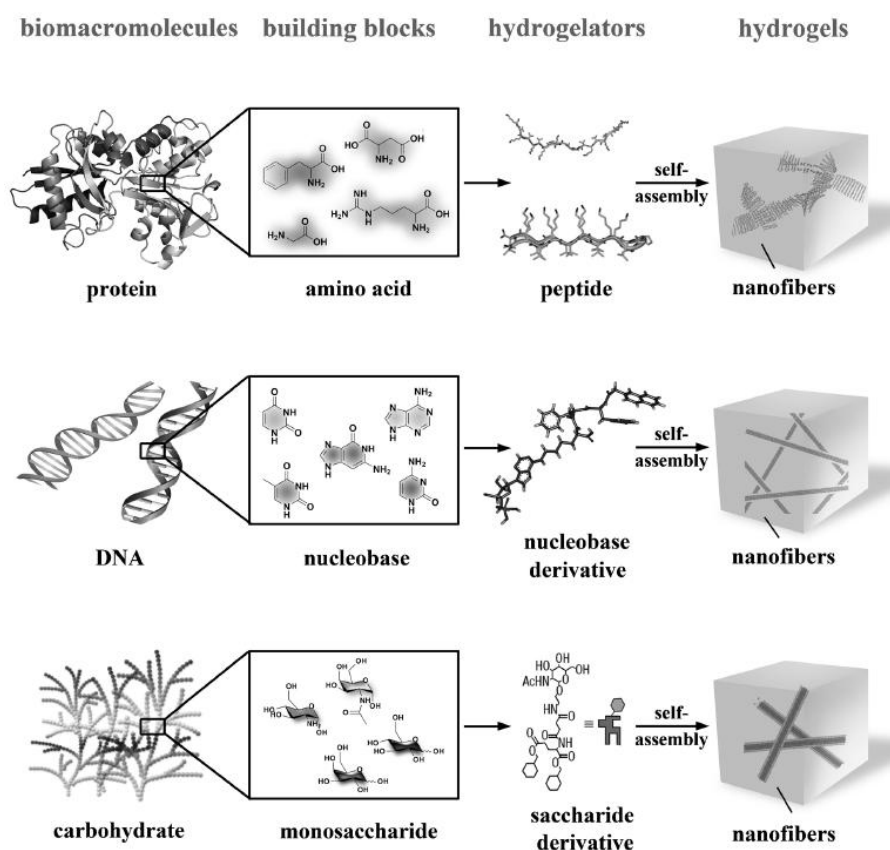


Figure 1. Supramolecular hydrogels are made from basic biological building blocks like amino acids, peptides, nucleic acid motifs, and saccharides that self-assemble into a 3D network through non-covalent interactions.

Among nucleotide building blocks, the self-assembly of 5'-Guanosine monophosphate (5'-GMP) is an archetypic example of

supramolecular polymerization. In the presence of sodium (Na^+) or potassium (K^+) ions, 5'-GMP organizes itself hierarchically to form various structures such as hydrogels, liquid crystals, nanofibers, and soluble aggregates.¹⁷ As a basic component of nucleic acids, 5'-GMP self-assembles via Hoogsteen hydrogen bonds to create noncovalent structures including G-monomer aggregates, G-dimers, and G-quadruplexes. These G-quartets are stabilized by hydrogen bonding and further stack through additional hydrogen bonds and π - π interactions, resulting in column-like G-quadruplex superstructures. This self-assembly is strongly enhanced in mildly acidic water, where protonated species and hydration water support the formation of GMP aggregates.^{18–20}

The spatial regulation of cellular functions arises from the localized activation of chemical processes within a complex, heterogeneous matrix. Developing synthetic macroscopic systems that replicate this ability enables fundamental investigations into how local molecular events give rise to functional properties at the macroscopic scale.²¹ Among various molecular building blocks, adenosine derivatives are particularly noteworthy, not only do they serve as the primary energy currency in biological systems, but they also exhibit amphiphilic hydrotropic behavior. Interestingly, the cellular concentration of ATP often exceeds immediate energy demands, reflecting its additional regulatory roles in maintaining protein stability, solubility, conformational flexibility, and aggregation behavior. Although the involvement of ATP and related nucleotides in preventing protein misfolding has only recently gained attention, numerous studies have already demonstrated the participation of other nucleotides, such as RNA, in droplet formation and protein aggregation.^{22–24} Consequently, exploring the ability of adenosine derivatives to form supra-molecularly polymerized fibrillar networks through the non-covalent co-assembly with other nucleotide building blocks represents a promising synthetic condensation strategy. Such systems could act as biomolecular nano-reactors, mimicking cellular functionality by

creating locally confined environments with high molecular concentration similar to those found within living cells.^{22,23,25}

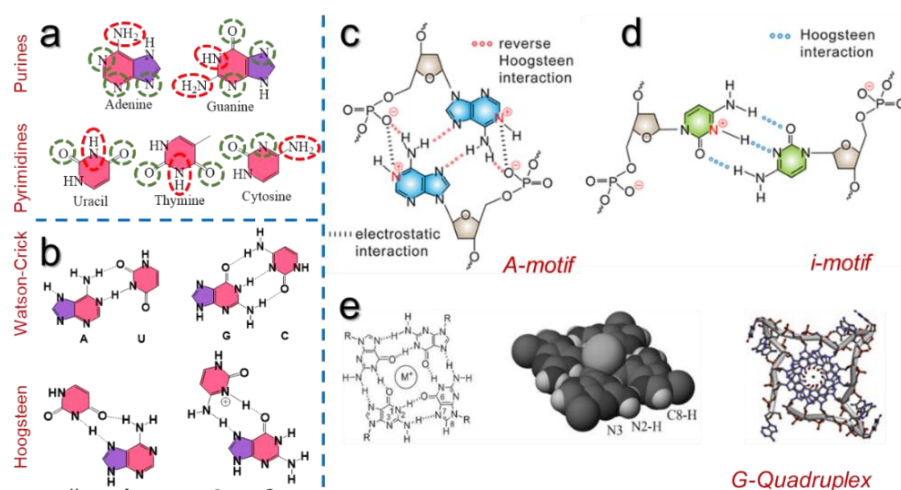


Figure 2. (a) Chemical structures of purine and pyrimidine bases, with hydrogen-bond donor sites highlighted in red and acceptor sites in green. (b) Representation of Watson-Crick and Hoogsteen hydrogen bonding patterns. (c) Molecular structure of the A:A base pair illustrating electrostatic interactions. (d) Molecular structure of the hemiprotonated C:C⁺ base pair and (e) the corresponding molecular structure and schematic representation of a G-quadruplex.²⁶

In this work, we present a minimalist “all-nucleotide” condensation approach that exploits cooperative interactions between guanosine 5'-monophosphate (GMP) and adenosine phosphate derivatives, namely AMP, ADP, and ATP, to form hierarchically organized supramolecular networks. GMP functions as the principal structural component by assembling into G-quadruplex architectures, whereas the adenosine phosphate derivatives regulate the assembly process through differences in phosphate number and inherent acidity. Although AMP couldn't induce this condensation process, both ADP and ATP promoted hierarchical biomolecular condensation. This spontaneous condensation behavior resembles a biogenic phase separation pathway and produces a concentration-dependent transformation from liquid-like droplets into fibrillar networks.^{15,27,28} Notably, at a higher level of self-assembly, the resulting ADP-GMP

(ADG) and ATP-GMP (ATG) fibrillar condensates evolved into self-supporting, stable hydrogels, offering physical evidence of extensive structural organization.

In contrast to conventional hydrogel-based catalytic systems, which often depend on immobilized enzymes or metal cofactors, the proposed “all-nucleotide” ADG/ATG platform acquires its catalytic function directly from the G4-rich framework. The stacked G4 domains generate a microenvironment that can stabilize reactive intermediates, thereby imparting intrinsic peroxidase-like activity to a fully metal-free and non-proteinaceous system. A major feature of this condensate/hydrogel system is its dual utility in biocatalysis. The ADG hydrogel serves as a biomimetic microenvironment, offering hydrated confinement for enzyme encapsulation and stabilization, while also acting as an active catalytic scaffold with enzyme-like function. To validate this, the hydrogel efficiently accommodates multiple enzymes, including β -galactosidase and glucose oxidase, supporting two- and three-step cascade reactions in which enzymatic conversions are effectively integrated with hydrogel-mediated catalysis. This confined environment promotes substrate channeling, minimizes diffusion constraints, and enhances overall reaction kinetics. By combining structural confinement with intrinsic catalytic activity in a minimalist platform, this system provides a versatile strategy for constructing biogenic artificial microreactors for complex biochemical transformations. Overall, the simplicity, modularity, and multifunctionality of the “all-nucleotide” hydrogel open new avenues for designing biomimetic catalytic systems based on condensation-driven self-assembly.

Chapter 2

Literature Review

Sarma et al. reported that non-covalent bio-condensation between DNA and guanosine monophosphate (GMP) mimics chromatin folding, yielding unique “all-nucleic” DNA-GMP condensates.¹⁵ GMP molecules form guanine tetrads via Hoogsteen hydrogen bonding, which stack through π - π interactions to generate G-quadruplex (G4) strands. DNA and GMP synergistically promote bio-condensate formation, with DNA inducing G4 assembly. The condensation mechanism was analyzed through kinetic spectroscopy, where enhanced negative ellipticity at 250 nm indicated G4 formation, and ThT fluorescence intensity reflected π - π stacking. Kinetic morphological evolution was probed using confocal microscopy, HR-TEM, FE-SEM, and AFM. Fluorescence imaging showed that at 10 μ M GMP, only intrinsic blue DNA fluorescence appeared; at 50 μ M, nanoaggregates emerged; at 90 μ M, nano-fibrillation initiated; and at 150 μ M, a uniform fibrillar DNA-GMP network with dominant green fluorescence formed. Microscopy confirmed that increasing GMP concentration drives the transition from coiled DNA strands to anisotropic nanostructures, progressing from spherical aggregates to helical fibrils. DNA wraps uniformly around G4 strands, forming fibrillar networks at higher GMP levels. These DNA-GMP condensates self-assemble into G4-based super-helical fibers, stabilized through synergistic hydrogen bonding and π - π stacking interactions.

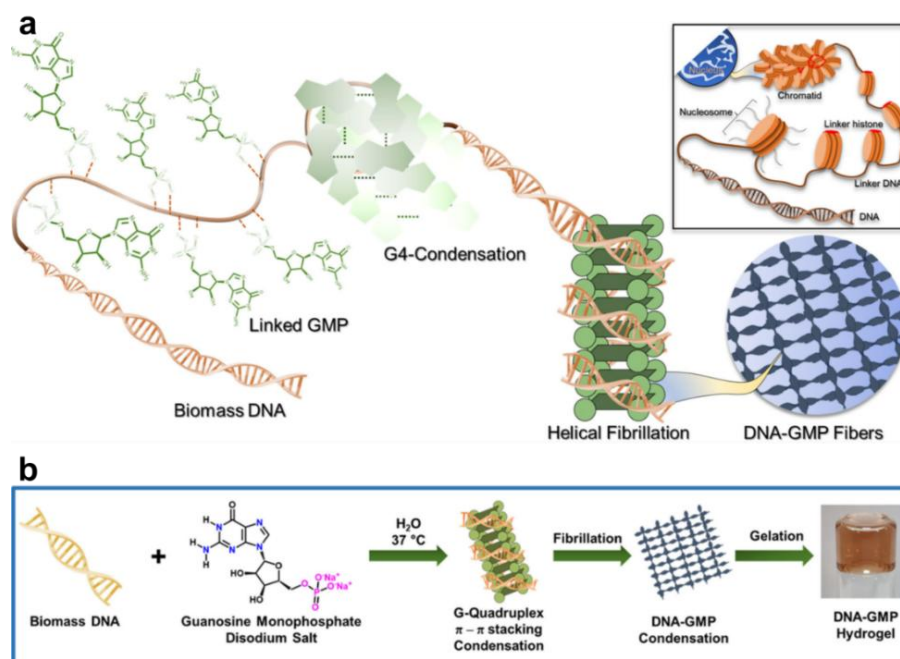


Figure 3. (a) DNA macromolecular assembled into a G4-condensate that leads to the formation of a helical fibrillar network, and **(b)** Transformation of DNA-GMP condensate to DNA-GMP hydrogel at higher order condensation.

In earlier studies, macromolecular DNA was shown to induce the formation of the G4 moiety, with DNA and GMP condensates acting as precursors for G-quadruplex-based superstructures. These superstructures further self-organize into super-helical fibers through synergistic hydrogen bonding and stacking interactions. Building on this, Thakur et al. explored an acidic amino acid, aspartic acid (AA), as a driver for GMP condensation and self-assembly, leading to the formation of G-quadruplex fibrillar nanozymes.²⁷ Spectroscopic analyses were employed to elucidate the assembly mechanism, revealing a negative Cotton band at 255 nm in the CD spectrum, indicative of antiparallel G-quadruplex (G4) formation. The ThT assay further confirmed G4 formation and $\pi-\pi$ stacking interactions. Zeta potential measurements ($\xi = -17.33$ mV) of AA-GMP condensates were lower than those of the individual components, suggesting interactions between the carboxyl groups of AA and the phosphate groups of GMP. Concentration-dependent pH titration demonstrated that AA's acidity

drives GMP self-assembly, with a concurrent rise in AA's pH and a drop in GMP's pH, indicating proton exchange. Microscopic analyses (FESEM, HRTEM) confirmed GMP-driven self-assembly: FESEM revealed a transition from spherical nanoaggregates to short nanofibers as GMP concentration increased, accompanied by the initiation of spherical voids. At higher concentrations, distinct voids surrounded by aligned nanofibers appeared, while HRTEM showed a uniform nanofibrillar network with periodic voids at the optimum concentration. CLSM indicated that low GMP concentrations produced spherical condensates, which merged into longer aggregates and evolved into protofibrils, ultimately forming a void-embedded fibrillar network with fluorescence localized exclusively within the fibrillar regions.

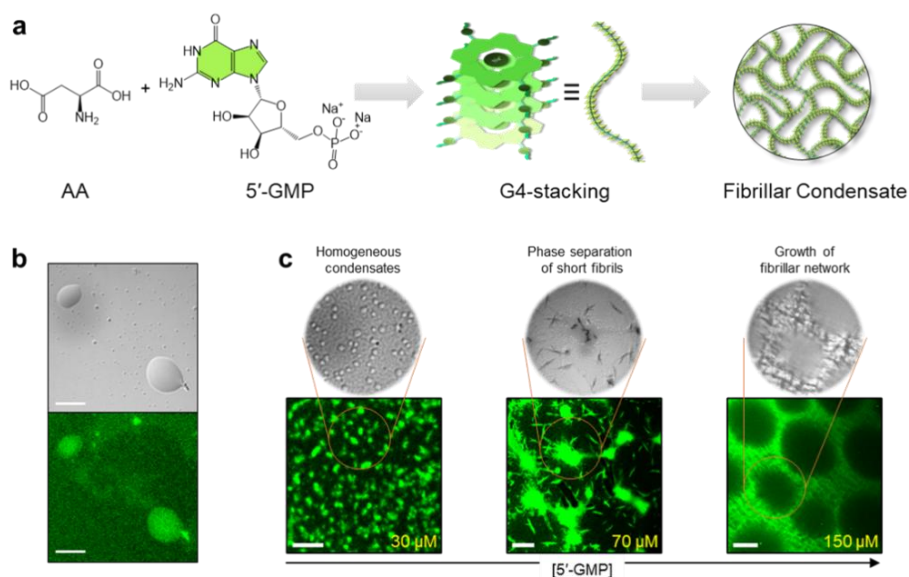


Figure 4. Acidic amino acid mediated phase separation promotes 5'-GMP self-assembly. **(a)** Chemical structures of aspartic acid and 5'-GMP, along with schematic representations illustrating G-quadruplex (G4) stacking and the ensuing supramolecular fibrillation. **(b)** Confocal microscopy images (DIC and green fluorescence channels) showing AA-GMP condensates during the initial condensation stage ($[GMP] = 10 \mu M$); scale bar: ~ 500 nm. **(c)** Morphological evolution from homogeneous condensates to phase-separated short fibrils and ultimately to nanofibrous networks with increasing 5'-GMP concentration; scale bar: $\sim 5 \mu m$.²⁷

Thus, from the above examples, it can be inferred that GMP condensation is facilitated by an acidic environment through noncovalent interactions, resulting in stacked G4 self-assembly. The acidity of the counter-molecules (either biomacromolecules or small biomolecules) protonates GMP phosphate groups, enabling hydrogen bonding between the counter-molecules and GMP. This leads to the formation of G4 stacks with Na^+ ion channels. This process is confirmed by the reduction in surface charge observed upon the condensation of GMP into G4 assemblies. Fibrillation occurs via π - π stacking and G4 templating, following a condensation pathway from spherical condensates to columnar G4 nanofibers. Unlike typical biomolecular stacking, 5'-GMP assembly is enthalpy-driven, primarily governed by cation binding, hydrogen bonding, and π - π interactions, rather than hydrophobic effects. Additionally, hydration water further modulates this acid-induced self-assembly by balancing solvation, confinement, and interactions between counter-molecules and GMP.

Two recent reports were comprehensively analyzed to elucidate the importance of adenosine-derived nucleotide building blocks. Dec et al. demonstrate that ATP acts as a molecular switch, modulating the liquid-liquid phase separation (LLPS) and amyloid fibrillization of chimeric peptides containing an amyloidogenic insulin-derived segment (ACC1-13) fused to oligolysine fragments of varying lengths.²³ Their findings reveal that ATP engages in selective and stoichiometric interactions with the oligolysine chains, resulting in divergent assembly pathways depending on chain length. For peptides with shorter oligolysine segments (K8, K16), ATP triggers direct and rapid fibril formation, bypassing the LLPS stage. Conversely, longer oligolysine stretches (K24, K32, K40) lead to ATP-mediated LLPS, evidenced by the formation of liquid droplets that subsequently mature into amyloid fibrils. Optical microscopy confirmed these liquid droplets in freshly mixed higher-order Kn co-aggregates, while fluorescence microscopy of ACC1-13K32 revealed that droplets persist for at least 30 minutes without significant enhancement of ThT fluorescence. Over time,

merging droplets correlated with increased ThT fluorescence in some droplets, with stochastic onset of fibrillization observed within these compartments. After 45 minutes, only select droplets exhibit ThT emission, suggesting heterogeneous nucleation events. As aggregation proceeds (up to 24 hours), a strong temporal correlation emerges between droplet growth and increased ThT fluorescence intensity, indicating fibril formation within droplets. Atomic force microscopy further corroborates amyloid fibril emergence, typically initiating at droplet interfaces. Consequently, ATP is shown to control the delicate balance between phase-separated droplet formation and fibril maturation via charge compensation and elevation of local biomolecular concentrations.

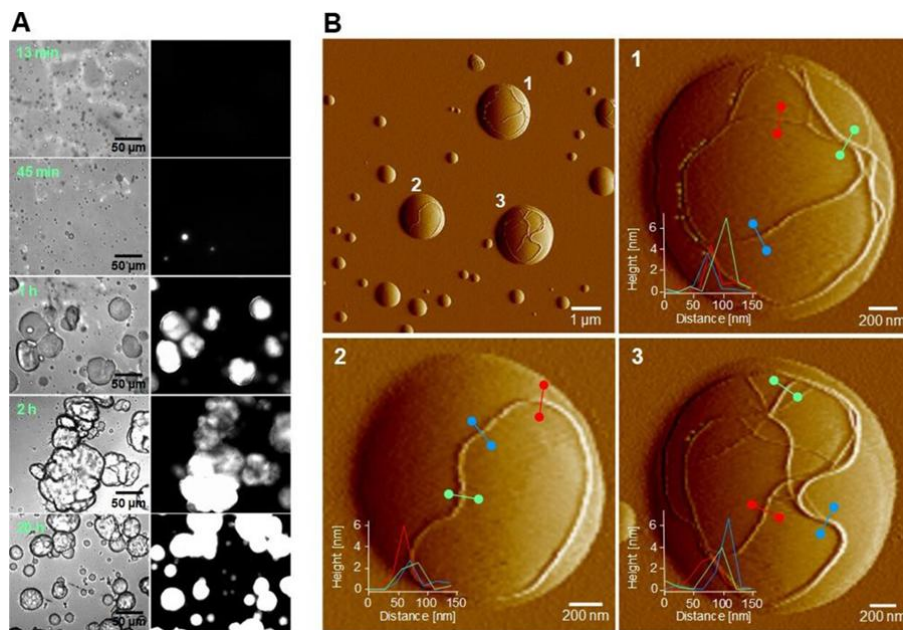


Figure 5. (A) Time-lapse microscopic images of a freshly mixed protein-ATP sample observed in the brightfield mode (left column) and ThT emission-enhancing fluorescence mode (right column). (B) AFM amplitude images capturing the intermediate LLPS-to-fibril structural transition. Images 1, 2, and 3 are the magnified sections of the image shown in the top left panel. Overlaid are cross-sections of selected specimen, as indicated by the likewise colored cross-lines, based on the corresponding height images.²³

A prior report by Dec et al. established that ATP can initiate both LLPS and fibrillization in chimeric peptides featuring oligolysine arms. Follow-up studies probed the reversibility and dynamic equilibrium of ATP–peptide assemblies under enzymatic modulation²². Employing apyrase, which enzymatically hydrolyzes ATP, the researchers observed dissolution of both LLPS droplets and fibrils, reaffirming ATP’s centrality in maintaining aggregate stability. Remarkably, proteinase K treatment induced a reversible fibril-to-droplet transition; cleaved peptide fragments with released ATP re-associated into droplets, revealing that ATP can both drive forward droplet-to-fibril conversion and, under appropriate conditions, enable fibril-to-droplet reversion. Furthermore, even minor stoichiometric shifts in ATP-to-peptide ratios destabilized fibrils, underscoring the crucial role of electrostatic interactions.

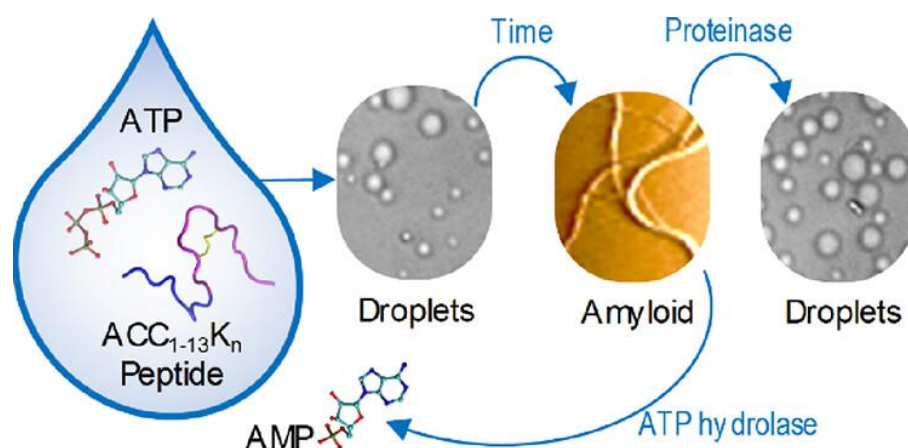


Figure 6. Dynamics of a protein-ATP system under enzymatic perturbation, showing an unusual reverse transition from fibrils to droplets.²²

Overall, these investigations extend the recognition of ATP beyond its canonical functions in bioenergetics and nucleic acid metabolism, identifying it as a potent amphiphilic hydrotrope present at millimolar intracellular concentrations, and a regulator of protein assembly, stability, dynamics, and aggregation propensity.

Multi-enzyme cascade reactions are central to mimicking natural biochemical pathways, where sequential enzymatic transformations

proceed in a coordinated and efficient manner. In such systems, the product of one enzyme serves as the substrate for the next, enabling complex reactions to occur with high specificity. However, artificial cascade systems often face significant challenges, including diffusion limitations, loss of enzyme activity, and inefficient transfer of intermediates between enzymes, which collectively reduce overall catalytic efficiency.²⁹

To overcome these limitations, metal–organic frameworks (MOFs), particularly ZIF-8, have been widely explored as platforms for enzyme immobilization. Their porous architecture and mild encapsulation conditions help preserve enzyme activity and enhance stability. A promising solution to this challenge is the development of peptide-induced super-assembly strategies that spatially organize enzyme-loaded MOF nanoparticles. In this approach, different enzymes such as glucose oxidase (GOx), horseradish peroxidase (HRP), and β -galactosidase are individually encapsulated within MOFs and then functionalized with complementary coiled-coil peptides. These peptides drive the assembly of discrete enzyme-containing particles into well-organized superstructures, effectively bringing multiple enzymes into proximity. This spatial arrangement facilitates rapid and direct transfer of intermediates between enzymes, significantly enhancing cascade efficiency through improved substrate channeling and reduced diffusion barriers.

Furthermore, the cascade system exhibits dynamic control through stimuli-responsive behavior. The peptide linkers responsible for super-assembly can be selectively cleaved by proteases, leading to disassembly of the structure and interruption of the cascade reaction. This enables a controllable “on/off” mechanism for enzymatic activity, adding an additional level of regulation. Overall, this strategy provides an advanced framework for designing efficient, tunable, and biologically inspired cascade systems with potential applications in biosensing, catalysis, and synthetic biology.

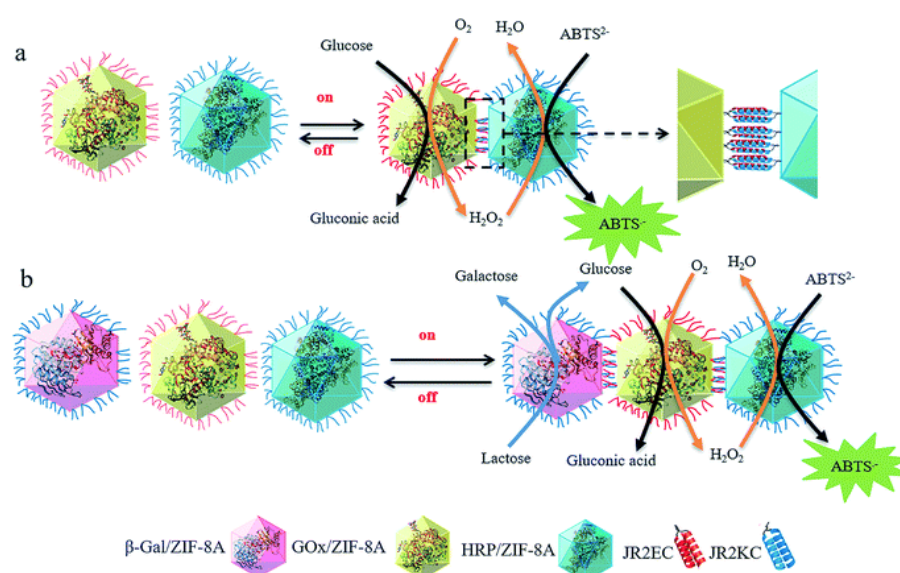


Figure 7. Schematic illustration of **(a)** two-enzyme (GOx/ZIF-8A and HRP/ZIF-8A) and **(b)** three-enzyme (β -gal/ZIF-8A, GOx/ZIF-8A and HRP/ZIF-8A) cascades “switch on” driven by complementary coiled-coil forming peptide (JR2EK and JR2KC) induced MOF superstructures and then “switch off” by chemically disrupting the coiled-coil superstructure.

Multienzyme cascade reactions play a crucial role in biological systems by enabling sequential transformations with high efficiency through direct transfer of intermediates between enzymes. Compared to conventional stepwise reactions, cascade systems minimize intermediate loss, eliminate purification steps, and enhance overall reaction rates. However, the development of artificial cascade systems is often limited by enzyme instability, diffusion barriers, and inefficient intermediate transfer, which reduce catalytic performance.³⁰

To address these challenges, materials such as hydrogels and metal–organic frameworks (MOFs) have been explored for enzyme immobilization. Hydrogels offer a biocompatible, cell-like environment that preserves enzyme structure and activity, making them suitable for cascade reactions, but their poor mechanical strength and limited stability under harsh conditions restrict practical use. In contrast, MOFs

like ZIF-8 provide high stability, tunable porosity, and protection, though they may constrain enzyme conformation and reduce activity.

To overcome these limitations, a hybrid hydrogel–MOF system (pAAm@ZIF-8) has been developed, where enzymes are encapsulated within a hydrogel core and shielded by an outer MOF layer. This synergistic design preserves enzyme activity while enhancing stability, leading to improved efficiency of the GOx–HRP cascade through better intermediate transfer and reduced diffusion loss.

Additionally, the system enables spatial organization of enzymes into distinct compartments, mimicking cellular architecture. This compartmentalization allows regulation of cascade pathways, including separation of competing enzymes like catalase, thereby controlling reaction flux and improving overall cascade performance.

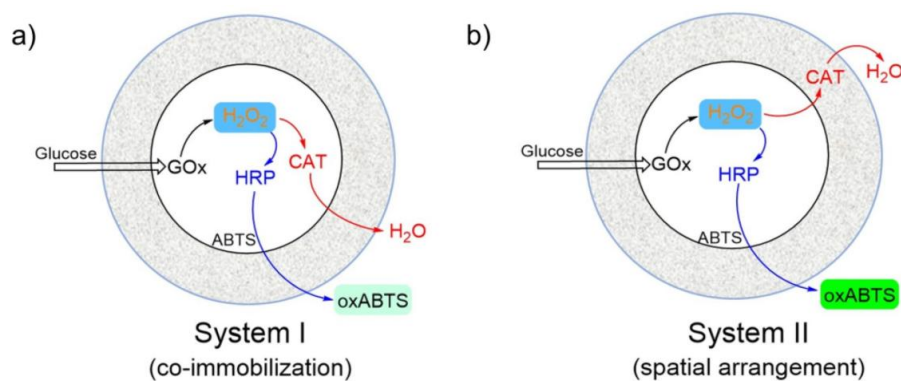


Figure 8. Incompatible cascade reactions in pAAm@ZIF-8. Schematic illustration of the co-encapsulation **(a)** and spatial arrangement **(b)** of CAT with GOx/HRP cascade in pAAm@ZIF-8.

Chapter 3

Motivation

Earlier studies have shown that G-quadruplex (G4) assemblies can be formed through the assistance of various biomolecular inducers, ranging from large macromolecules like DNA to smaller entities such as acidic amino acids. While these systems have significantly advanced our understanding of supramolecular self-assembly, their potential in functional applications, particularly in multi-step biocatalysis, remains an area of growing interest. In biological systems, cascade reactions rely on the efficient transfer of intermediates between sequential enzymes, enabling high reaction specificity and minimal loss of intermediates. However, replicating such efficiency in artificial systems is challenging due to diffusion limitations, poor spatial organization, and instability of enzymes. Supramolecular G4-based materials, owing to their ordered structure, tunable microenvironment, and ability to confine biomolecules, offer a promising platform to overcome these limitations and facilitate cascade reactions.

Motivated by this, we sought to design a minimal and fully nucleotide-based supramolecular system capable of supporting cascade enzymatic processes. By utilizing guanosine 5'-monophosphate (GMP) as a structural unit, we aimed to construct a biogenic scaffold that not only promotes G4 assembly but also provides a confined and organized environment for sequential enzymatic transformations. Such confinement can enhance substrate channeling, improve catalytic efficiency, and stabilize enzyme activity.

Furthermore, incorporating small nucleotide-derived molecules, such as adenosine phosphate derivatives, introduces an additional level of functionality to the system. These molecules can actively modulate both the self-assembly process and the local microenvironment, thereby influencing enzymatic activity and stability. This approach effectively bridges the gap between structural self-assembly and functional

biocatalysis, enabling the development of simple, tissue-like soft materials that mimic natural cellular environments. By leveraging such nucleotide-based components, the resulting supramolecular networks can provide confined and interactive spaces that enhance substrate channelling and improve the overall efficiency of cascade reactions. Ultimately, this strategy presents a minimalistic yet powerful platform for designing advanced biohybrid materials tailored for efficient and sustainable cascade biocatalysis.

Chapter 4

Objectives

- **To develop a facile synthetic methodology:** We aim to design a simple and efficient strategy to form an all-nucleotide-based condensate using non-covalent interactions such as hydrogen bonding and π - π stacking, avoiding complex chemical modifications.
- **To understand the assembly mechanism:** We investigate how different nucleotides interact and what specific role each component plays in the formation of the condensate, using spectroscopy studies and advanced microscopy techniques.
- **To transform into a functional hydrogel:** We explore the conversion of the condensate into a stable bio-hybrid hydrogel with enhanced structural integrity, responsiveness to external conditions, and improved catalytic properties.
- **To apply in cascade biocatalysis:** Finally, we evaluate the potential of this system as a microreactor for multi-step enzymatic cascade reactions, aiming to improve efficiency through better confinement and substrate channelling.

Chapter 5

Experimental Section

5.1. Materials

All chemicals used in the experiments are of analytical grade and were used as received without further purification. All experiments were conducted at ambient conditions. Adenosine triphosphate disodium salt (purity 98%), Adenosine diphosphate disodium salt (purity 95%), Adenosine monophosphate disodium salt (purity 98%) and Guanosine monophosphate disodium salt (purity 98%), 3',5,5'-tetramethylbenzidine (TMB), D-glucose, and lactose monohydrate were purchased from Sisco Research Laboratory, India. Hydrogen peroxide (30% v/v), ethanol, sodium acetate, acetic acid, sodium chloride, magnesium chloride, monobasic sodium phosphate, dibasic sodium phosphate, and Tris base were obtained from Merck (India). The enzymes β -galactosidase from *Escherichia coli* (Grade VIII, lyophilized powder) and glucose oxidase from *Aspergillus niger* (Type VII, lyophilized powder) were purchased from Sigma-Aldrich. Thioflavin T (ThT) dye was purchased from TCI chemicals. MilliQ water was used throughout the experiments.

5.2. Methods

UV-visible spectroscopy: UV-vis absorption spectra were recorded using a Cary 60 spectrometer in the wavelength range of 220-320 nm with a quartz cuvette of 10 mm path length. The GMP concentration was systematically varied from 10 μ M to 100 μ M, while the concentrations of AMP, ADP, and ATP were maintained at 100 μ M.

CD spectroscopy: Circular dichroism (CD) spectra were recorded using a JASCO J-815 spectropolarimeter in the wavelength range of 200-340 nm with a quartz cuvette of 1 mm path length. The spectra were scanned at a rate of 50 nm min⁻¹, and baseline corrections were carried out using Milli-Q water. The GMP concentration was systematically varied from

10 μ M to 100 μ M, while the concentrations of AMP, ADP, and ATP were maintained at 100 μ M.

Th-T Assay: Steady-state fluorescence spectra were recorded on a Horiba JobinYvon Fluoromax-4P spectrofluorometer (model FM-100). All measurements were conducted using samples at their respective condensate concentrations.

ATR-FTIR spectroscopy: FTIR spectra were recorded using a Bruker Alpha II spectrometer equipped with an ATR accessory. The GMP concentration was systematically varied from 10 to 100 mM, while the concentrations of AMP, ADP, and ATP were maintained at 100 mM.

Zeta Potential Measurements: Zeta potential measurements were performed using a Nanoplus Zeta Potential Analyzer equipped with a quartz flow cell to determine the surface charge of the pristine molecules and their condensates. The condensates were prepared at a concentration of 1 mM and incubated for 12 hours along with the corresponding pristine biomolecules to ensure complete surface formation.

pH titration: The condensates were prepared at a total concentration of 1 mM by varying the GMP content while keeping the concentrations of AMP, ADP, and ATP constant. The pH of the condensates was measured using a LABMAN LM10 pH meter.

Microscopic Characterizations: Confocal laser scanning microscopy (CLSM) was carried out using an Olympus IX-83 microscope equipped with a multiline Ar gas laser (405 nm excitation). Field-emission scanning electron microscopy (FESEM) was performed on Au-coated, dried AA-GMP condensates prepared at different concentrations and temperatures using a Carl Zeiss Supra 55 microscope.

Samples for microscopic imaging were prepared at a total concentration of 100 μ M by varying the GMP content. For CLSM imaging, 5 μ M ThT was added to the samples and incubated for 1 hour to allow self-assembly. The samples were then drop-cast onto cleaned cover slides and incubated at 30 $^{\circ}$ C for further self-assembly and drying.

Rheological Study: Rheological measurements of the synthesized hydrogels were performed using an Anton Paar Physica MCR 301 rheometer. Dynamic strain sweep tests were conducted at a constant angular frequency of 10 rad.s^{-1} , while frequency sweep experiments were carried out at a fixed strain amplitude of 1% at 25 °C. Thixotropic hysteresis loop measurements were recorded by varying the strain from 1% to 500% at a constant angular frequency of 10 rad.s^{-1} for each time interval.

Enzyme Kinetics and Catalytic Assay: Enzyme kinetics and cascade biocatalytic assays were evaluated by monitoring time-dependent changes in absorbance using a Varian Cary 100 Bio UV–visible spectrophotometer equipped with a quartz cuvette ($\approx 10 \text{ mm}$ path length). Electron paramagnetic resonance (EPR) measurements were carried out on a JEOL JES-FA200 ESR spectrometer immediately after introducing H_2O_2 into a pre-mixed solution of the hydrogel and DMPO. Hydroxyl radical generation was further assessed using a terephthalic acid (TA) fluorescence assay on the same fluorometer, where hydrogel, TA, and H_2O_2 were mixed and incubated for 20 minutes prior to recording the fluorescence spectra.

5.3. Synthetic Methods

Synthesis of the Condensates

A clear, homogeneous solution forms upon mixing $100 \text{ }\mu\text{M}$ adenosine-derived nucleotides (AMPNa_2 , ADPNa_2 , and ATPNa_2) with $100 \text{ }\mu\text{M}$ GMPNa_2 in water. After incubation at 30 °C for 1 hour, the formation of condensates in the ADP-GMP (ADG) and ATP-GMP (ATG) mixtures is visually confirmed using a red laser pointer, which reveals a distinct Tyndall effect. For comparative studies, condensates were similarly prepared with varying GMP concentrations and subsequently analysed using various spectroscopic and microscopic techniques. Each condensation process was freshly prepared before

every experiment to ensure the stability of the metastable states formed during condensation.

Synthesis of the hydrogels

To transform the ADG and ATG condensate to hydrogels, 100 mM of each component was dissolved in 1 mL of water. The mixture was then stirred and gently heated with a heat gun until it became a clear, transparent solution. This solution was left undisturbed for 10 minutes, allowing the ADG and ATG hydrogels to form. The resulting hydrogels were subsequently used for measuring their mechanical properties.

Peroxidase-Mimetic Activity Evaluation

Peroxidase activity of the ADP-GMP hydrogel was assessed using the H₂O₂/TMB system, comprising 100 µL of 10× diluted hydrogel, 2300 µL acetate buffer (0.01 M, pH 4.4), 300 µL H₂O₂ (100 mM), and 300 µL TMB (10 mM). The catalytic oxidation of TMB was monitored by measuring the absorbance at 652 nm. Steady-state kinetic parameters were obtained from two experimental sets: (1) varying TMB concentrations (0.05–1.20 mM) at fixed H₂O₂ (10 mM) and hydrogel levels, and (2) varying H₂O₂ concentrations (1–12 mM) at fixed TMB(1mM) and hydrogel concentrations. All measurements were performed at room temperature.

Kinetic parameters were calculated according to the Michaelis–Menten model:

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

where V_{max} represents the maximum reaction velocity, $[S]$ denotes the substrate concentration, K_m is the Michaelis–Menten constant, and V corresponds to the initial velocity.

Evaluation of the specific activities (SA)

Kinetic assays were conducted as follows: acetate buffer (1 mL, 0.01 M, pH 4.4) was supplemented with varying nanozyme concentrations, fixed

H₂O₂ (10 mM), and TMB (1 mM). The resulting mixtures were analyzed to obtain kinetic data. Specific activity was calculated by using the equation:

$$SA = \frac{\left[\frac{v}{(\varepsilon \times l)} \times \left(\frac{\Delta A}{\Delta t} \right) \right]}{m}$$

where V is the total reaction volume (μL); ε is the molar absorptivity of TMB (39,000 M⁻¹cm⁻¹), l is the light path length (cm), ΔA/Δt is the initial rate of change in absorbance; m is the weight (mg) of enzymes.

Enzymatic Mechanism Elucidation and Hydroxyl Radical Detection

Hydroxyl radical (•OH) generation was examined using electron paramagnetic resonance (EPR) spectroscopy with DMPO (1 mM) as the spin-trapping agent. The experimental conditions were maintained identical to the kinetic assays, except that DMPO was used in place of TMB. Following a 10 min incubation, the reaction mixture was transferred into a quartz capillary and immediately subjected to EPR analysis.

Further quantification of •OH generation was carried out using a terephthalic acid (TA) fluorescence assay. In this method, 0.5 mL of terephthalic acid solution (1 mM in 1 M NaOH) was mixed with 4.3 mL of buffer containing H₂O₂ (10 mM) and 100 μL of hydrogel, and the mixture was incubated for 15 min. Fluorescence measurements were performed with excitation at 315 nm, and emission spectra were recorded over time to monitor the formation of the fluorescent hydroxylated product.

Enzymatic Cascade Synthesis and Catalytic Activity

Enzymatic cascade reactions were performed under mild, physiologically relevant conditions. A stock solution of glucose oxidase (GOx, 1 mg mL⁻¹) was prepared in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) containing 150 mM NaCl. β-Galactosidase (β-Gal, 500 U mL⁻¹) was prepared in Tris–HCl buffer (0.05 M, pH 7.0) supplemented with 100 mM NaCl and 2 mM MgCl₂. ADG hydrogels were synthesized

as described earlier, and enzyme solutions were incorporated during gel formation to obtain a final hydrogel volume of 1 mL. For the two-enzyme cascade, GOx was encapsulated at an optimized concentration of 0.1 mg mL⁻¹, whereas for the three-enzyme cascade, β -Gal (50 U mL⁻¹) and GOx (0.1 mg mL⁻¹) were co-encapsulated within the hydrogel matrix. The enzyme-loaded hydrogels were then aged for 12 h to ensure effective encapsulation and stabilization.

Catalytic reactions were initiated by adding 100 μ L of the enzyme-loaded hydrogel to 3 mL of acetate buffer (0.01 M, pH 4.4) containing TMB (1 mM) and varying substrate concentrations: glucose (0.025-0.5 mM) for the two-step cascade and lactose (0.025–0.5 mM) for the three-step cascade. The reaction mixtures were thoroughly mixed and incubated at 25 °C for 1 h. The formation of oxidized TMB was monitored by measuring absorbance at 652 nm. Calibration plots were constructed based on the linear relationship between absorbance and substrate concentration, and the limits of detection (LOD) for glucose and lactose were determined accordingly.

Chapter 6

Results and Discussions

6.1. Condensation of Nucleotides

Earlier reports have demonstrated the remarkable self-assembly behaviour of 5'-GMP, which forms diverse noncovalent superstructures such as G-monomer aggregates, G-dimers, and G-quadruplexes through Hoogsteen hydrogen bonding and π - π stacking interactions. The incorporation of Na^+ ions within the GMP macrocyclic stacks contributes to exceptional structural stability and facilitates spontaneous condensation. This self-association of monomeric 5'-GMP into supramolecular polymeric architectures is further promoted in mildly acidic aqueous environments, where protonated acidic groups and hydration water molecules assist in stabilizing the G-macrostructure.^{31–}

33

Literature reports suggest that adenosine phosphate derivatives, such as ATP and ADP, can induce a mildly acidic environment.³⁴ To investigate the effect of these derivatives on the self-assembly behaviour of GMP, pristine biomolecules were mixed in equimolar ratios and incubated to facilitate the formation of GMP-based assemblies. Interestingly, the resulting mixtures exhibited uniform condensation. Quantitatively, mixing aqueous solutions of 100 μM each of disodium salts of adenosine-phosphate derivatives (AMP, ADP, and ATP) with 100 μM 5'-GMP- Na_2 yielded homogeneous transparent solutions that subsequently underwent phase separation, leading to the formation of colloidal assemblies, as confirmed by the observation of Tyndall scattering (Figure 9).

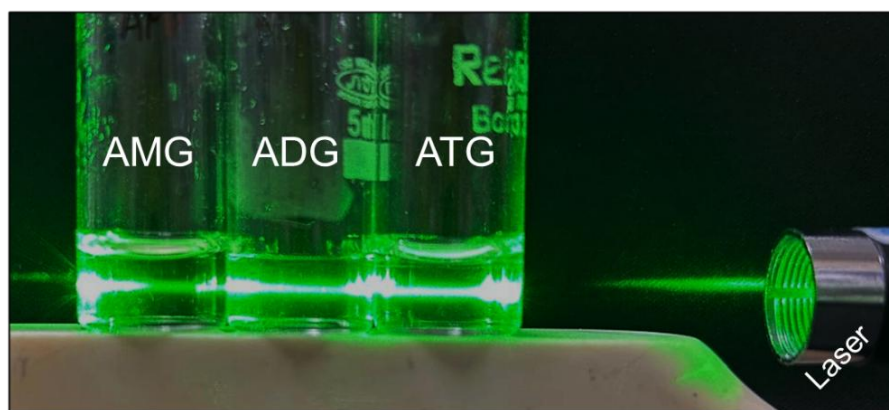
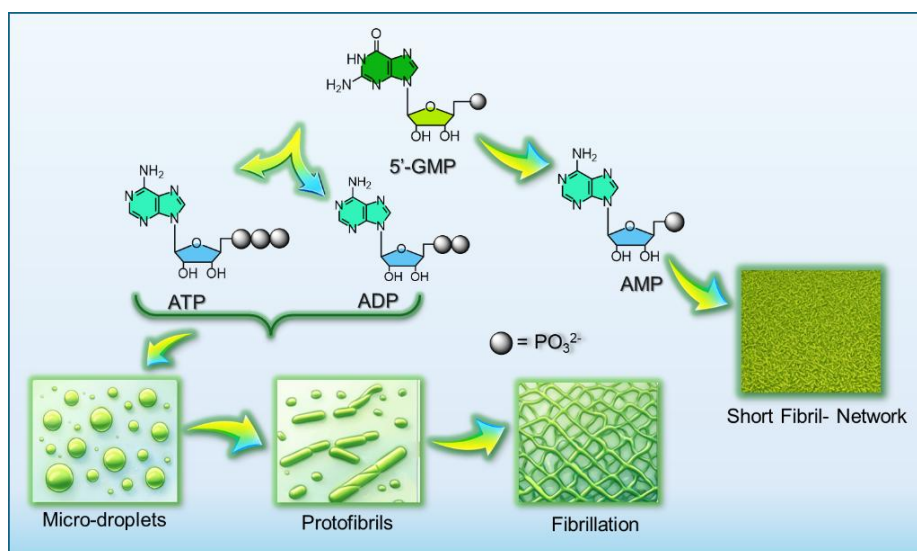


Figure 9. Tyndall effect for the visual confirmation of AMG, ADG, and ATG condensate formation, showing the laser beam passing through the condensate vial.

Further, to verify the condensation mechanism and enable a comparative analysis, a series of concentration-dependent studies was performed. To gain deeper insight into the condensation process, the concentration of GMP was varied from 10 μM to 100 μM while maintaining the concentration of adenosine phosphate derivatives at 100 μM , since GMP self-assembly predominantly governs the condensation behaviour. For comparative evaluation, detailed spectroscopic and microscopic analyses were conducted using fixed concentrations of the adenosine phosphate derivatives (AMP, ADP, and ATP).



Scheme 1: Schematic representation of the condensation of GMP with AMP, ADP and ATP.

6.2. Kinetic Morphological Studies

Scanning electron microscopy (SEM) of the condensates revealed well-defined entangled fibrillar networks in both the ADP-GMP (ADG) and ATP-GMP (ATG) systems (Figure 10a, 10b). In contrast, the AMP-GMP (AMG) system exhibited only short and sparsely distributed fibrillar structures (Figure 10c). These observations indicate that condensation in the ADG and ATG systems leads to more pronounced and homogeneous fibril formation between the nucleotide pairs, confirming the occurrence of an efficient condensation process. Notably, the degree of entanglement was higher in the ATG system compared to ADG, suggesting that the number of phosphate groups influences the extent and organization of fibrillation. These findings motivated further investigation into the kinetics and morphological evolution of fibril growth in these systems.

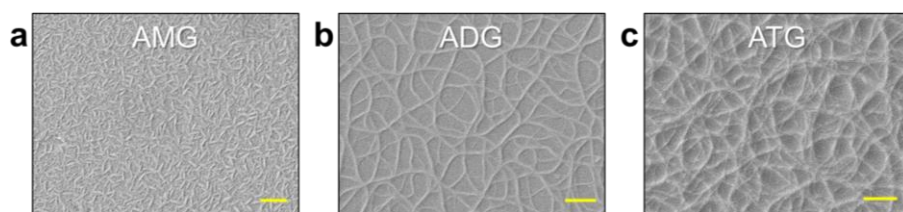


Figure 10. FE-SEM micrographs of the condensates **(a)** AMG, **(b)** ADG and **(c)** ATG systems. (sample concentration $\sim 100 \mu\text{M}$); scale bar: $1 \mu\text{m}$.

To better understand the self-assembly mechanism and the non-covalent interactions governing the condensation of ADP and ATP with GMP, concentration-dependent aggregation kinetics were investigated using a combination of microscopic techniques. Given the crucial role of GMP in promoting G-quadruplex (G4) formation and hierarchical fibrillation, the morphological evolution was examined across a range of GMP concentrations while keeping the concentrations of the adenosine phosphate derivatives constant. At a low GMP concentration of $10 \mu\text{M}$, droplet-like condensates were prominently observed in both ADG and ATG systems, indicating the onset of liquid-liquid phase separation (Figure 11a,e; 12a,e). Upon increasing the GMP concentration to $30 \mu\text{M}$, these droplets aggregated into larger clusters in both systems (Figure 11f, 12f). In the ADG system, the aggregated droplets transitioned into short protofibrils at $50 \mu\text{M}$ GMP (Figure 11b,g), while the ATG system displayed the emergence of fractal-like morphologies, reflecting a higher degree of structural organization (Figure 12b,g). Further increasing the GMP concentration to $70 \mu\text{M}$ resulted in the formation of small fractal-like networks in the ADG system (Figure 8c,h) and denser, more interconnected fractal structures in the ATG system (Figure 9c,h). At an optimal GMP concentration of $100 \mu\text{M}$, both systems exhibited a well-developed, homogeneous fibrillar network, with the ATG system showing a slightly more entangled architecture (Figure 11d,i; 12d,i).

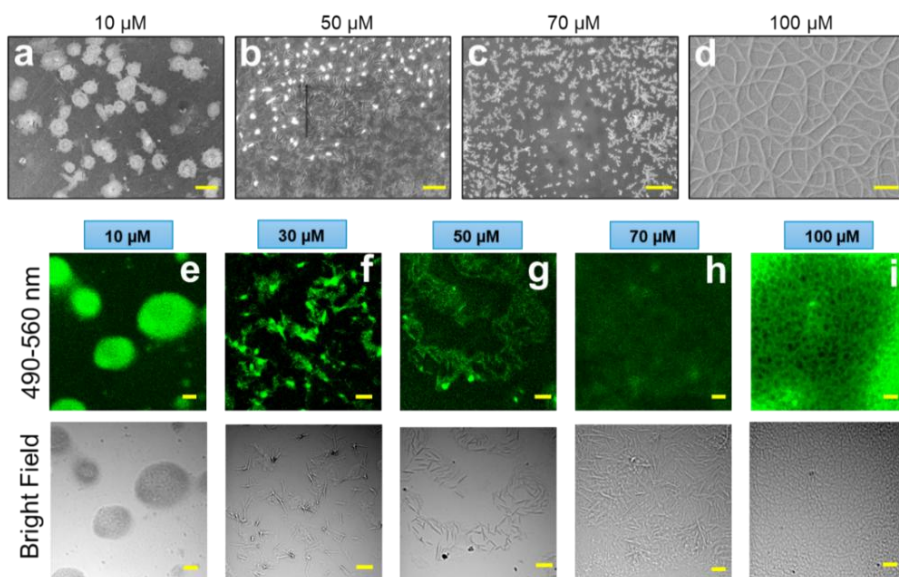


Figure 11. Kinetic morphological evolution of the ADG system with varying GMP concentrations, illustrating the transition from droplet-like condensates to fibrillar networks. FESEM (**a-d**) and CLSM (**e-i**) micrographs show: (**a,e**) 10 μM GMP, formation of droplet-like assemblies; (**f**) 30 μM GMP, aggregation of droplets; (**b,g**) 50 μM GMP, emergence of protofibrils; (**c,h**) 70 μM GMP, development of fractal-like structures; and (**d,i**) 100 μM GMP, formation of an interconnected fibrillar network. FESEM: (Scale bar $\sim 1 \mu\text{m}$) and CLSM: (Scale bar $\sim 5 \mu\text{m}$).

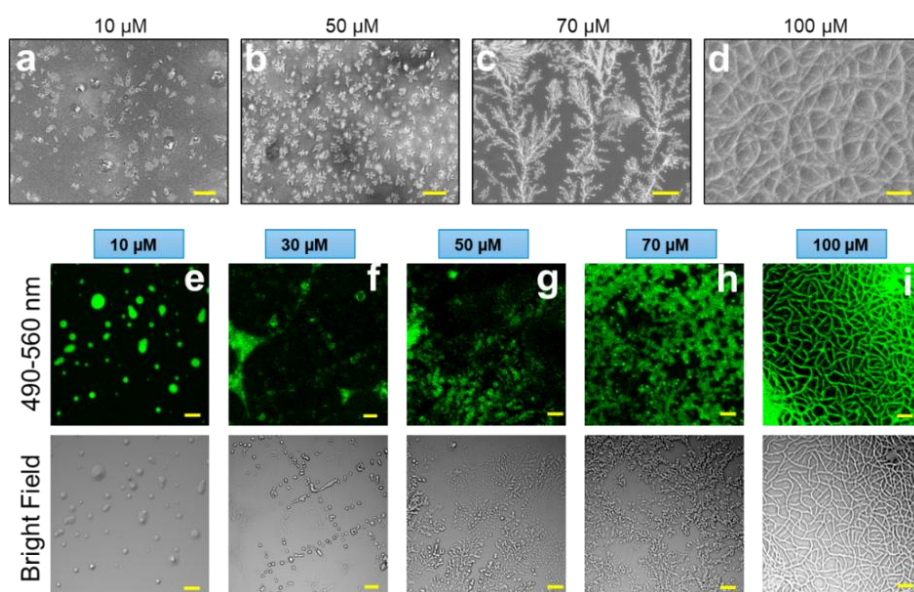


Figure 12. Kinetic morphological evolution of the ATG system with varying GMP concentrations, illustrating the transition from droplet-like

condensates to fibrillar networks. FESEM **(a-d)** and CLSM **(e-i)** micrographs show: **(a,e)** 10 μM GMP, formation of droplet-like assemblies; **(f)** 30 μM GMP, aggregation of droplets; **(b,g)** 50 mM GMP, emergence of protofibrils; **(c,h)** 70 μM GMP, development of fractal-like structures; and **(d,i)** 100 μM GMP, formation of an interconnected fibrillar network. FESEM: (Scale bar $\sim 1 \mu\text{m}$) and CLSM: (Scale bar $\sim 5 \mu\text{m}$).

6.3. UV-vis Spectroscopy

The UV-vis spectrum of GMP displayed characteristic absorption peaks at 251 nm with a shoulder at 275 nm, corresponding to $\pi\text{-}\pi^*$ transitions within the guanine base. In contrast, adenosine phosphate derivatives (AMP, ADP and ATP) exhibited a peak at 258 nm arising from $\pi\text{-}\pi^*$ transitions in the adenosine base. The observed spectral shifts, primarily due to $\pi\text{-}\pi^*$ transitions, remained evident after the interaction, indicating the persistence of non-covalent interactions (Figure 13a,b,c).³⁵

To elucidate the stoichiometric ratio governing the assembly, the concentration of adenosine phosphate derivatives was maintained constant while that of GMP was systematically varied. The absorption band of GMP ($\lambda_{\text{max}} \sim 251 \text{ nm}$) showed a gradual bathochromic shift upon incremental addition of adenosine phosphate derivatives, signifying the occurrence of non-covalent interactions and the subsequent formation of a stable supramolecular assembly.

Job's plot analysis further confirmed a 1:1 stoichiometric ratio between GMP and each adenosine phosphate derivative (100 mM each), consistent with the formation of a well-defined supramolecular complex (Figure 13b,d,f).³⁶

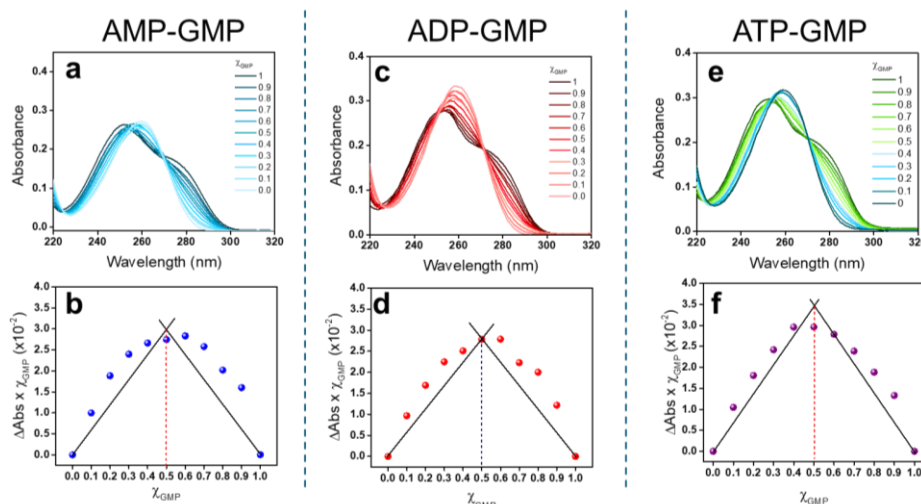


Figure 13: UV-vis spectroscopic analysis and Job's plot for (a,b) AMP-GMP, (c,d) ADP-GMP, and (e,f) ATP-GMP systems. The GMP concentration was systematically varied from 10 to 100 μM , while the concentrations of AMP, ADP, and ATP were fixed at 100 μM .

6.4. Circular Dichroism Spectroscopy

For a deeper understanding of G-assembly formation and the associated chirality of adenosine-phosphate derivatives, systematic circular dichroism (CD) analyses of the condensates were performed. Initially, the chirality of the pristine biomolecules and that of the condensates at a 1:1 stoichiometric ratio was examined, as this ratio was found to be optimal for condensation. In the CD spectrum of the AMG condensate, no significant ellipticity was observed, indicating the absence of ordered assembly (Figure 11c). In contrast, the ADG and ATG condensates displayed CD signals characteristic of antiparallel G-quadruplex structures, featuring two positive Cotton bands near 300 nm and 225 nm, and a signature negative Cotton band around 260 nm.³⁷ These elliptic bands, evident in both ADG and ATG condensates but absent in the pristine biomolecules (as shown in Figure 14a,b), confirm the formation of a G-quartet-like architecture. Furthermore, the absence of any additional chiral signals corresponding to adenosine-derived self-assemblies (such as A-motif structures) indicates that the condensation process is predominantly governed by 5'-GMP molecules, without the emergence of separate adenosine-based assemblies.²⁶

To evaluate the extent of G-quadruplex formation, a systematic concentration-dependent CD analysis was performed on the ADG and ATG condensates by varying GMP concentration. The CD spectra revealed a progressive increase in characteristic G4 ellipticity with rising GMP concentration, indicating enhanced G-assembly formation (Figure 14e,f). However, at a 1:1 stoichiometric ratio of ADP:GMP and ATP:GMP, the condensates exhibited optimal chiroptical responses, corresponding to the most efficient condensation. These observations suggest that the GMP concentration plays a critical role in regulating the condensation process and governs the overall assembly mechanism.^{15,27}

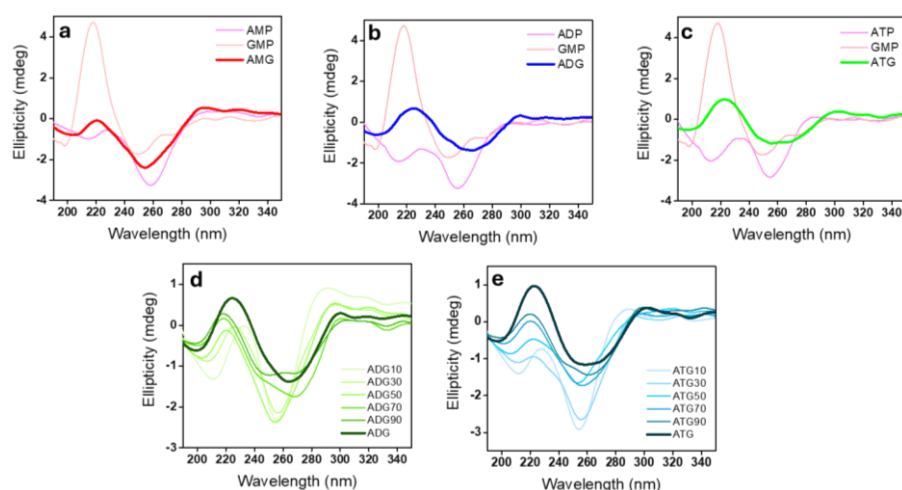


Figure 14: Circular dichroism (CD) spectra of nucleotide components and their condensates: **(a)** AMP, GMP, and AMP-GMP condensate (AMG); **(b)** ADP, GMP, and ADP-GMP condensate (ADG); **(c)** ATP, GMP, and ATP-GMP condensate (ATG). Concentration-dependent CD spectra of **(d)** ADG and **(e)** ATG recorded by varying GMP from 10 to 100 μ M, while ADP and ATP concentrations were fixed at 100 μ M.

6.5. ATR-FTIR Spectroscopy

As the condensation process involves an entirely nucleic system, the phosphate functionalities of both nucleotide molecules play a crucial role in mediating condensation and subsequent fibril formation. To probe the involvement of the phosphate groups during this process, ATR-FTIR analysis was performed. GMP exhibited two characteristic PO_3^{2-} stretching bands, with symmetric and asymmetric stretches at 978

and 1086 cm^{-1} , respectively. Similar phosphate signatures were observed for AMP, ADP, and ATP. AMP displayed a single PO_3^{2-} stretching band at 1080 cm^{-1} , while ADP showed two distinct peaks at 1079 and 1224 cm^{-1} . ATP exhibited multiple phosphate-related bands at 910, 951, 1073, and 1239 cm^{-1} .^{22,23,38}

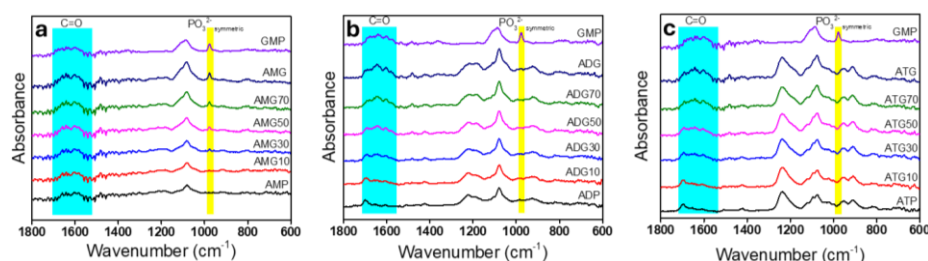


Figure 15: ATR-FTIR spectra: **(a)** AMP, GMP, AMP-GMP condensate (AMG); **(b)** ADP, GMP, ADP-GMP condensate (ADG); **(c)** ATP, GMP, ATP-GMP condensate (ATG) by varying GMP concentration from 10-100 mM, keeping AMP, ADP and ATP concentrations maintained at 100 mM.

When AMP was combined with GMP under condensation conditions, the characteristic phosphate bands of GMP remained unaltered, indicating minimal interaction between AMP and GMP phosphate groups. In contrast, the addition of ADP or ATP led to the disappearance of GMP's phosphate bands, suggesting that ADP and ATP actively participate in the condensation process by engaging with the phosphate groups of GMP (Figure 15a-c).

Additionally, ATR-FTIR analysis revealed a distinct C=O stretching band at 1642 cm^{-1} in GMP, absent in ADP and ATP due to the lack of a carbonyl group. Upon increasing the concentration of GMP in ADP or ATP solutions, a pronounced and broadened band appeared at 1642 cm^{-1} , indicating the formation of a more rigid and ordered supramolecular structure.

6.6. ThT Assay

The degree of π - π stacking and guanosine (G)-driven self-association was quantitatively analyzed using a Thioflavin T (ThT)

fluorescence assay. ThT, a well-established fluorogenic probe, binds to ordered supramolecular assemblies through noncovalent interactions such as end stacking, groove binding, or intercalation within GMP channels (Figure 16a). These interactions restrict the intramolecular rotational freedom of ThT, stabilizing it in a more rigid and planar conformation. As a result, nonradiative relaxation pathways are suppressed, leading to a significant enhancement in fluorescence intensity.

Fluorescence emission spectra of ThT-loaded condensates, recorded at an excitation wavelength of 350 nm, showed a characteristic emission peak around 450 nm (Figure 16b). At an optimized concentration (100 μ M), comparative analysis revealed that the fluorescence intensity followed the trend: ADG > ATG > AMG (Figure 16d). Although the relative fluorescence enhancement (FL/FL_0 , compared to free ThT) was similar for ADG and ATG systems, the AMG condensate exhibited a considerably lower enhancement, indicating minimal π - π stacking. This observation is consistent with earlier circular dichroism (CD) and FTIR results.

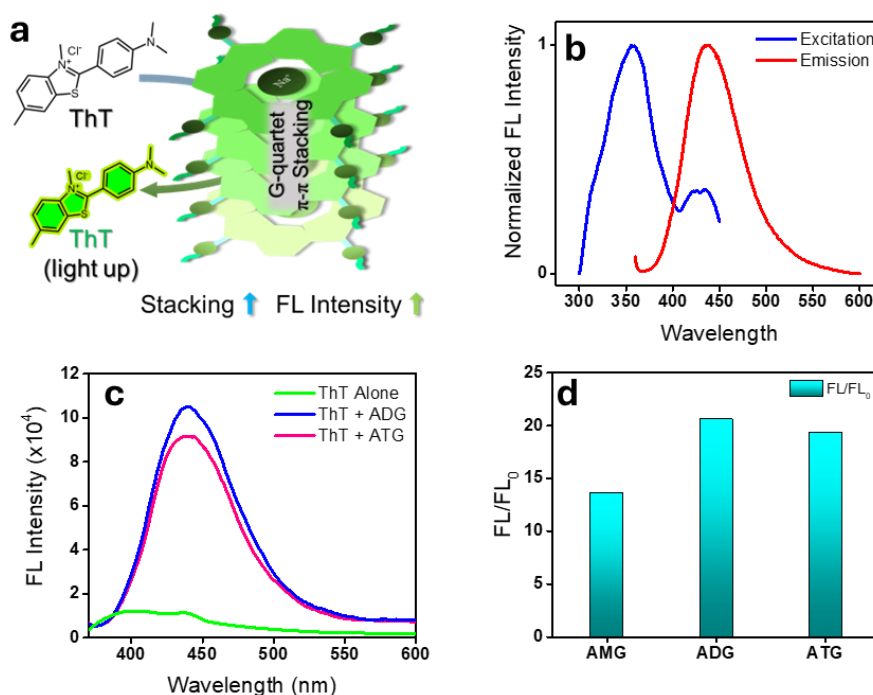


Figure 16. (a) Schematic representation of ThT binding within G-quadruplex channels. (b) Fluorescence excitation and emission spectra

of Thioflavin T (ThT)-loaded ADG condensates, showing an excitation maximum at 350 nm and an emission maximum at ~450 nm ($\lambda_{\text{ex}} = 350$ nm). **(c)** Comparison of fluorescence emission spectra of pristine ThT and ThT-loaded condensates, demonstrating enhanced emission upon binding to fibrillar assemblies. **(d)** Relative fluorescence enhancement (FL/FL₀) compared to free ThT, showing ThT incorporated in AMG, ADG, and ATG condensates at an optimal concentration of 100 μM .

To further elucidate the role of GMP in governing stacking interactions, concentration-dependent fluorescence studies were performed, providing insight into the evolution of supramolecular organization. The AMG system exhibited nearly invariant fluorescence intensity across the GMP concentration range (10-100 μM), indicating the absence of significant changes in molecular packing or electronic coupling. This behavior suggests that AMP lacks the structural features required to promote effective π - π stacking or G-quartet formation, thereby preventing the development of extended ordered assemblies (Figure 27a).

In contrast, both ADG and ATG systems displayed a linear increase in fluorescence intensity with increasing GMP concentration, reflecting a progressive enhancement in stacking interactions. This trend can be attributed to the concentration-dependent formation of G-quadruplex (G4) structures, where higher GMP availability facilitates the assembly of guanine bases into planar G-quartets stabilized by Hoogsteen hydrogen bonding and π - π stacking. The linearity of the fluorescence response further indicates a cooperative and hierarchical self-assembly process, in which initial nucleation events are followed by elongation into extended fibrillar or network-like architectures. Such behavior strongly supports that GMP acts as the primary driving force for the formation of ordered supramolecular frameworks in ADG and ATG systems (Figure 17b,c).

Temperature-dependent fluorescence measurements provided additional insight into the stability of these assemblies. Upon increasing

temperature, a gradual decrease in fluorescence intensity was observed for all systems, consistent with the thermal disruption of non-covalent interactions, particularly π - π stacking and hydrogen bonding within G-quartet structures (Figure 17e). However, the extent of fluorescence reduction differed significantly between systems. The ADG system retained comparatively higher fluorescence intensity than ATG, indicating enhanced thermal stability. This suggests that ADG forms more compact and strongly interacting stacked architectures, likely due to more efficient G4 stacking, improved inter-fibrillar interactions, or a higher degree of structural ordering. The greater resistance of ADG to thermal perturbation highlights the presence of a more robust and well-organised supramolecular network.

To decouple the specific role of GMP from that of adenosine phosphate derivatives, control experiments were conducted by varying the concentrations of ADP and ATP while maintaining a constant GMP level. Only marginal increases in fluorescence intensity were observed under these conditions, indicating that ADP and ATP contribute minimally to the stacking interactions. Their role is therefore likely limited to secondary effects, such as modulating the local environment, influencing electrostatic interactions, or facilitating molecular proximity, rather than directly participating in π - π stacking or G-quartet formation (Figure 17d).

Furthermore, across the entire concentration range investigated, ADG consistently exhibited higher fluorescence intensity than ATG. This observation suggests that ADP is more effective than ATP in supporting the formation of ordered assemblies, possibly due to differences in steric hindrance, charge distribution, or molecular flexibility. ATP, with its additional phosphate group, may introduce increased electrostatic repulsion or steric constraints that hinder optimal stacking and packing of G-quartets, thereby leading to comparatively less ordered structures.

Overall, these results clearly demonstrate that the AMG system lacks the ability to form hierarchical self-assembled networks due to insufficient stacking interactions. In contrast, ADG and ATG systems undergo GMP-driven hierarchical assembly into stacked architectures, with ADG forming the most stable, densely packed, and structurally well-defined network. The findings highlight the critical role of GMP concentration in dictating the extent of G4 formation and emphasize how subtle differences in molecular components can significantly influence the organization, stability, and functional properties of supramolecular systems.

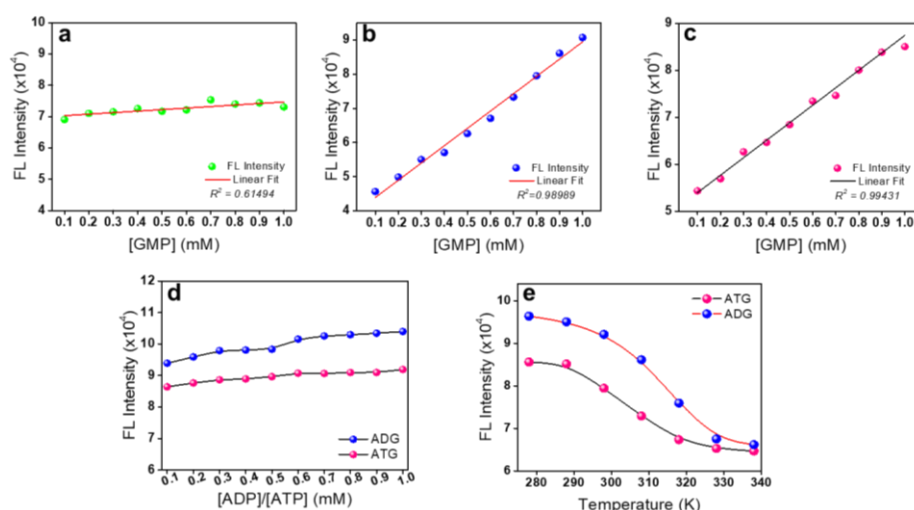


Figure 17. (a-c) Fluorescence responses of AMG, ADG, and ATG condensates, respectively, as a function of increasing GMP concentration. (d) Concentration-dependent fluorescence emission of ADG and ATG condensates with varying ADP and ATP concentrations, respectively, at a fixed GMP concentration (100 μ M). (e) Temperature-dependent fluorescence showing decreased intensity due to thermal disruption of G-quartet stacking, with ADG exhibiting greater stability than ATG.

6.7. pH Titration

Since 5'-GMP can self-assemble into columnar G-quadruplex (G4) stacks under mildly acidic conditions, the pH of the fibrillation-inducing environment plays a pivotal role in governing the condensation process.

To elucidate the influence of pH on this phenomenon, a systematic, concentration-dependent pH titration was carried out. The initial pH measurements of the nucleotide precursors revealed marked differences: ADP and ATP exhibited acidic pH values of 3.7 and 3.62, respectively, whereas AMP displayed a near-neutral pH of 6.32. Consequently, only ADP and ATP inherently provide the mildly acidic milieu favourable for G4 formation and subsequent fibrillar condensation, consistent with earlier experimental observations.

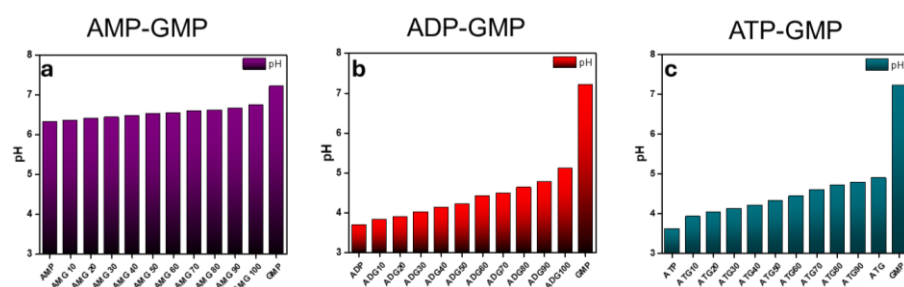


Figure 18: pH titration of the condensates. **(a)** AMP, GMP, AMP-GMP condensate (AMG); **(b)** ADP, GMP, ADP-GMP condensate (ADG); **(c)** ATP, GMP, ATP-GMP condensate (ATG) by varying GMP concentration from 0.1-1 mM, keeping AMP, ADP and ATP concentrations maintained at 1 mM.

Upon gradual addition of GMP, the pH of the reaction mixtures increased progressively, with the final pH values for the ADG and ATG systems reaching 5.11 and 4.9, respectively (Figure 18a-b). These trends indicate an ongoing proton redistribution between the participating nucleotides. During condensation, protons are exchanged as the acidic environment of ADP or ATP is partially neutralized by the basic sites of GMP, resulting in a net rise in pH. This dynamic proton exchange supports a cooperative interaction between the nucleotide components that facilitates the self-assembly and stabilization of the G4-based condensates.

6.8. Zeta Potential Analysis

To assess the impact of surface charge on condensation, zeta potential measurements were performed. The pristine nucleotides GMP,

AMP, ADP, and ATP exhibited negative surface potentials in the range of -25 to -35 mV, consistent with phosphate-rich surface functionalities. Following condensation, ADG and ATG assemblies showed a pronounced increase in zeta potential, whereas the AMG system displayed only a modest change (Figure 19a-c). This behaviour suggests that higher-order condensation of GMP into G4 networks promotes the formation of positively charged Na^+ ion channels and an ordered, columnar arrangement of GMP phosphate groups. Consequently, extensive condensation of GMP, particularly with ADP and ATP, reduces the overall negative surface charge, shifting the zeta potential toward neutrality.

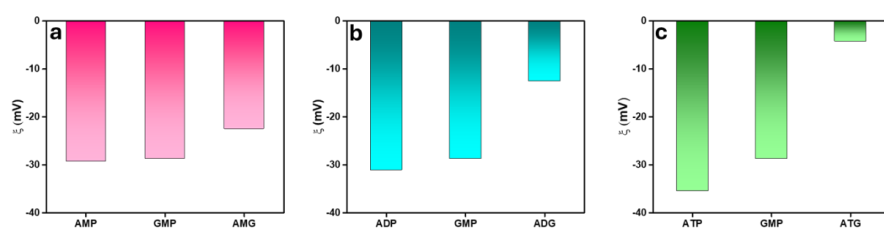


Figure 19: Zeta potential analysis of nucleotide condensates: **(a)** AMP-GMP condensate (AMG), **(b)** ADP-GMP condensate (ADG), and **(c)** ATP-GMP condensate (ATG), compared to their respective monomeric nucleotides AMP, ADP, ATP, and GMP, each at 1 mM concentration.

6.9. Self-assembly mechanism

These results collectively elucidate a highly cooperative and finely tuned condensation mechanism governed by the interplay of π - π stacking, multivalent phosphate interactions, and pH-dependent modulation. GMP acts as the principal structural scaffold, self-assembling into well-defined G-quadruplex (G4) architectures through a combination of planar π - π stacking between guanine bases and Hoogsteen hydrogen bonding. This intrinsic propensity of GMP to form ordered stacks provides the foundation for higher-order supramolecular organization.

The presence of ADP and ATP introduces an additional level of control through their multivalent phosphate groups and associated

acidity. These nucleotides do not directly integrate into the G4 core but instead modulate the surrounding microenvironment. Their higher negative charge density enhances local electrostatic interactions, while their ability to lower the pH promotes protonation equilibria favourable for stabilizing G4 stacking. Notably, ADP induces more efficient and stable higher-order assembly compared to ATP, suggesting that subtle differences in phosphate number and resulting pH shifts critically influence the optimal window for G4 formation and stacking fidelity (Figure 20).

Mechanistically, the assembly follows a hierarchical pathway. Initially, weak multivalent interactions and concentration-dependent molecular crowding drive liquid-liquid phase separation (LLPS), resulting in the formation of dynamic, micron-scale droplets. Within these confined condensates, the local concentration of GMP increases significantly, facilitating nucleation of protofibrillar G4 structures. These protofibrils subsequently elongate and interconnect to form dense fibrillar networks, ultimately giving rise to gel-like or semi-solid supramolecular architectures. This transition from liquid droplets to structured networks reflects a maturation process driven by progressive strengthening of non-covalent interactions.

Spectroscopic and fluorescence analyses further clarify the roles of individual components. GMP concentration directly dictates the extent and efficiency of π -stacking and G4 formation, effectively controlling the structural density and order of the network. In contrast, adenosine derivatives (ADP and ATP) primarily function as environmental regulators, tuning parameters such as pH, ionic strength, and electrostatic screening, rather than acting as structural building blocks. This distinction underscores a division of roles between structural drivers and modulatory agents within the system.

In comparison, AMP exhibits significantly reduced condensation behaviour. Its lower phosphate content results in diminished charge density and weaker electrostatic contributions, while its near-neutral pH

fails to create conditions conducive to efficient proton-mediated stabilization of G4 assemblies. Consequently, AMP-containing systems display limited phase separation and yield poorly organized or transient structures.

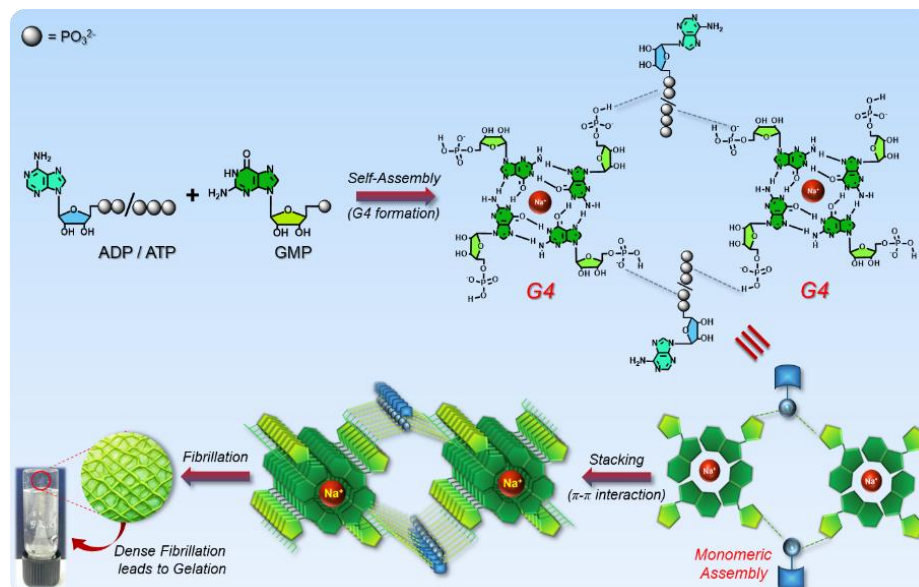


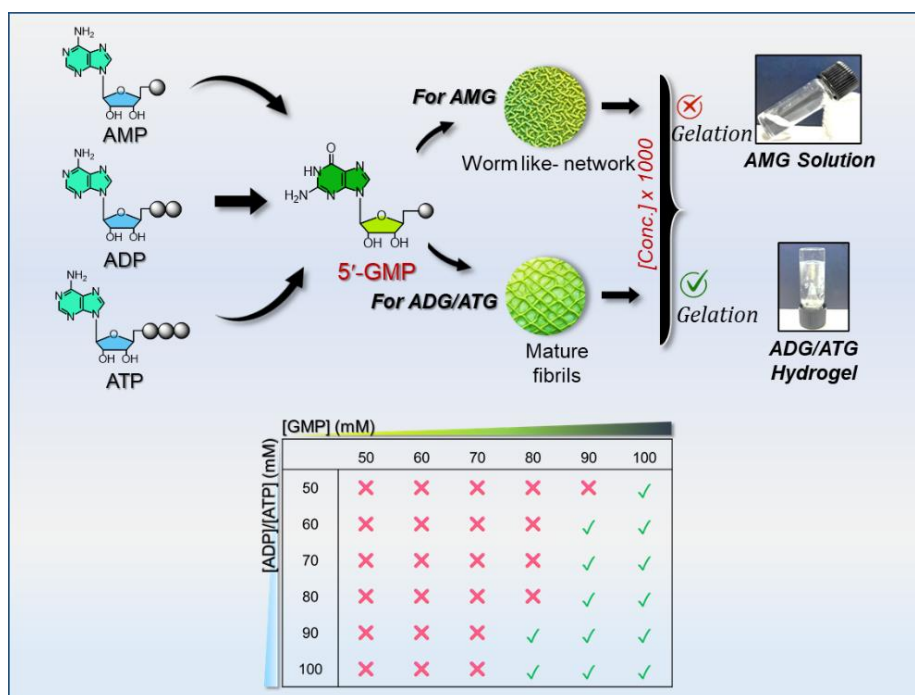
Figure 20. Proposed mechanism illustrating GMP-driven G-quadruplex formation and π - π stacking, assisted by multivalent phosphate interactions, resulting in hierarchical fibrillar assembly and hydrogel formation.

Taken together, this study reveals a GMP-dominated, phosphate-assisted, and pH-regulated assembly pathway that enables the formation of robust, hierarchical “all-nucleotide” supramolecular networks. The findings not only provide mechanistic insight into nucleotide-driven self-assembly but also highlight how subtle variations in molecular composition and environmental conditions can be leveraged to precisely control structure, stability, and function in biomimetic systems.

6.10. ADG and ATG extensive condensation leading to hydrogelation

At higher levels of molecular organization, fibrillar nanostructures often undergo a transition into cohesive hydrogel states. In our system, the bio-condensation of ADP, ATP, and GMP generated distinct fibrillar assemblies under dilute conditions, motivating a deeper investigation

into their self-assembly behaviour at elevated concentrations. Remarkably, when the condensation was carried out using 100 mM ADP/ATP and 100 mM GMP, the system underwent a spontaneous transformation into a mechanically stable hydrogel. This concentration-dependent reorganization highlights the inherent ability of nucleotide-based assemblies to evolve into higher-order supramolecular architectures. In contrast, the AMP-GMP system does not exhibit such hierarchical organization; although fibrillar structures are formed, they remain in a liquid-like state even at optimal concentrations and fail to transition into a hydrogel. In contrast, the AMG system, exhibiting disordered worm-like or loosely distributed structures, did not achieve stable gelation and persisted as a weakly aggregated solution. Conversely, both ADG and ATG systems displayed strong gelation across a broad concentration range, with minimum gelation thresholds of 50 mM for ADP/ATP and 80 mM for GMP (Figure 6b). This wide gelation regime is likely driven by enhanced fibrillar assembly at elevated GMP concentrations, where densely interconnected networks promote hydrogel formation through efficient percolation. Furthermore, the increased density of fibrillar networks at higher concentrations strengthens intermolecular interactions, leading to improved mechanical stability of the gels. This structural organization also facilitates better entanglement and crosslinking within the network, thereby enhancing the overall rigidity and integrity of the hydrogel matrix (Scheme 2).



Scheme 2. Schematic representation of the transformation of a fibrillar condensate (AMG) to a homogeneous transparent hydrogel at higher order self-assembly (ADG, ATG). Diagram depicting gelation (✓) and non-gelation (✗) as a function of concentration, highlighting the critical threshold required for hydrogel formation.

6.11. Rheological Analysis

To quantitatively assess the mechanical properties of the ADG and ATG hydrogels, rheological analyses were performed. Dynamic strain-sweep experiments (Figure 21a) demonstrated a linear viscoelastic region at low shear strains ($\leq 100\%$), where the storage modulus (G') and loss modulus (G'') exhibited parallel and constant values, indicating the hydrogels' ability to withstand deformation while maintaining structural integrity. Notably, the ATG hydrogel displayed slightly higher G' and G'' values compared to ADG, reflecting enhanced mechanical strength. Frequency sweep measurements further confirmed the viscoelastic behaviour of both hydrogels across the tested range ($0.1\text{-}100\text{ rad}\cdot\text{s}^{-1}$), with G' consistently exceeding G'' , characteristic of predominantly elastic networks (Figure 21b). These rheological findings collectively

indicate robust physical integrity, high strain tolerance, and excellent viscoelastic properties of the ADG and ATG hydrogels. Kinetic, morphological, and spectroscopic investigations consistently identified GMP as the key structural component responsible for driving fibrillar network formation in both ADG and ATG systems. To further substantiate its role in gelation, systematic concentration-dependent studies were carried out by varying the GMP content. At lower concentrations (<80 mM), both systems remained in a sol state, indicating insufficient intermolecular interactions for network formation. A distinct sol-to-gel transition was observed at approximately 80 mM GMP, representing the critical gelation threshold. Upon further increasing the GMP concentration beyond this point, robust hydrogel formation was achieved, with maximum gel strength observed at 100 mM GMP in both systems (Figure 21 c,d).

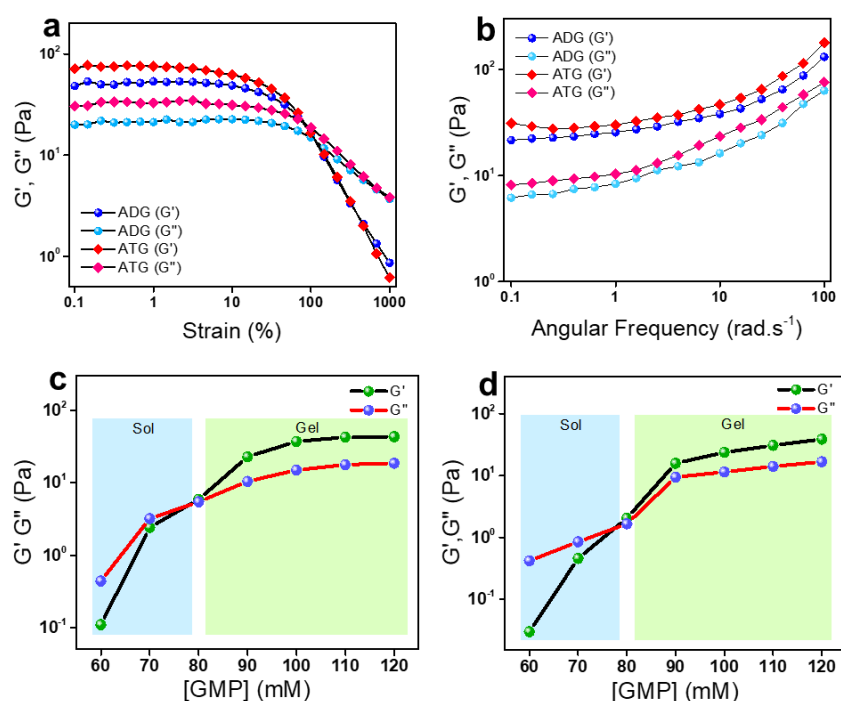


Figure 21. Rheological and mechanical characterization of ADG and ATG hydrogels. **(a)** Amplitude sweeps measurements at a constant angular frequency of 10 rad.s^{-1} , **(b)** Frequency sweep measurements at constant 1% strain. **(c, d)** GMP concentration-dependent rheological analysis indicating the minimum and optimal gelation concentrations for ADG and ATG systems.

6.12. Intrinsic Peroxidase Mimicking Activity

Phase separation drives the formation of dense condensates that locally enrich catalytic species and substrates, creating confined microenvironments that mimic enzyme-like reaction kinetics. By enhancing molecular proximity and reactant collision frequency, such compartmentalized assemblies promote efficient catalytic turnover and provide a useful platform for biomimetic catalysis³⁹.

Peroxidase-mimicking systems are especially attractive in this context because they combine oxidative catalytic activity with greater stability and tunability than natural peroxidases. Their integration with phase-separated condensates can further improve catalytic efficiency through reactant enrichment and reduced diffusion barriers, supporting applications in biomedicine, environmental remediation, and biosensing⁴⁰.

The ADP-GMP hydrogel was used as a catalytic microreactor for the oxidation of chromogenic substrates 3,3',5,5' tetramethylbenzidine (TMB) to ox-TMB in the presence of H₂O₂ (Figure 22a). Monomeric constituents (ATP, ADP, and GMP) were also evaluated to assess their catalytic performance. However, the ADP-GMP hydrogel exhibited significantly enhanced kinetic behaviour, displaying a higher $V_{\max}$ compared to the individual monomeric components as well as the ATP-GMP hydrogel system. This observation highlights the catalytic advantage imparted by the confined microenvironment and macromolecular organization within the ADP-GMP hydrogel network, which likely facilitates improved substrate accessibility and catalytic turnover (Figure 22b). To comprehensively confirm peroxidase-mimicking activity, the catalytic oxidation of chromogenic substrates was evaluated by monitoring the time-dependent increase in absorbance due to the formation of oxidised products. For TMB, the generation of the characteristic oxidized radical cation (TMB^{•+}) was tracked at 652 nm. The progressive increase in absorbance over time verified efficient catalytic oxidation and supported the peroxidase-like nature of the

system (Figure 22c). A control experiment was also performed with/without a catalyst to confirm the effect of the catalytic microenvironment. Upon addition of 1 mM TMB and 10 mM H_2O_2 to the 10 mM ADP-GMP fibrillar condensate (10-fold diluted hydrogel), a distinct absorption peak emerged at 652 nm, indicating the oxidation of TMB to $\text{TMB}^{\bullet+}$. In contrast, no such peak was observed for TMB alone or for the TMB/ H_2O_2 mixture in the absence of ADP-GMP, confirming the intrinsic peroxidase-like activity of the ADP-GMP system (Figure 22d).

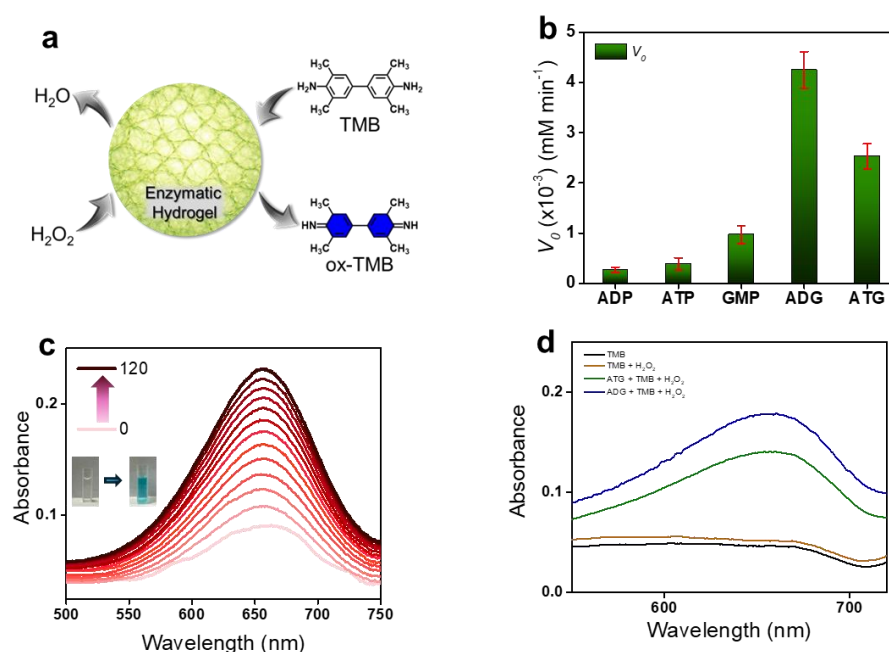


Figure 22. Peroxidase-like activity of ADP-GMP hydrogel: **(a)** Schematic of the intrinsic peroxidase-like catalysis. **(b)** Controlled kinetic parameter with respect to the native biomolecules ADP and GMP (inset showing the kinetic graphs). **(c)** Time-derived oxidation of TMB to ox-TMB. **(d)** Controlled experiments for the different conditions of the peroxidase-like activity.

Kinetic analysis of the peroxidase-mimicking activity of the ADP-GMP hydrogel was performed by varying the concentration of TMB (0.05-1.0 mM) and H_2O_2 (1-12 mM) while monitoring the time-dependent change in absorbance at 652 nm (Figure 17a,b). For both substrates, the reaction profiles exhibited a typical Michaelis-Menten behavior, indicating that the hydrogel-catalyzed oxidation process

follows enzyme-like saturation kinetics. As the substrate concentration increased, the initial reaction rate increased correspondingly until approaching a plateau, consistent with the progressive saturation of the available catalytic sites within the hydrogel network.

To further quantify the catalytic performance, the kinetic data were analysed using the Lineweaver–Burk double-reciprocal model, from which the Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were determined (Figure 17c,d). The K_m value provides insight into the apparent substrate affinity of the catalytic system, with lower values indicating stronger substrate interaction, whereas V_{max} reflects the maximum catalytic rate achieved at substrate saturation. Together, these parameters offer a quantitative assessment of the peroxidase-like efficiency of the ADP-GMP hydrogel and enable direct comparison with other natural and artificial catalytic systems.

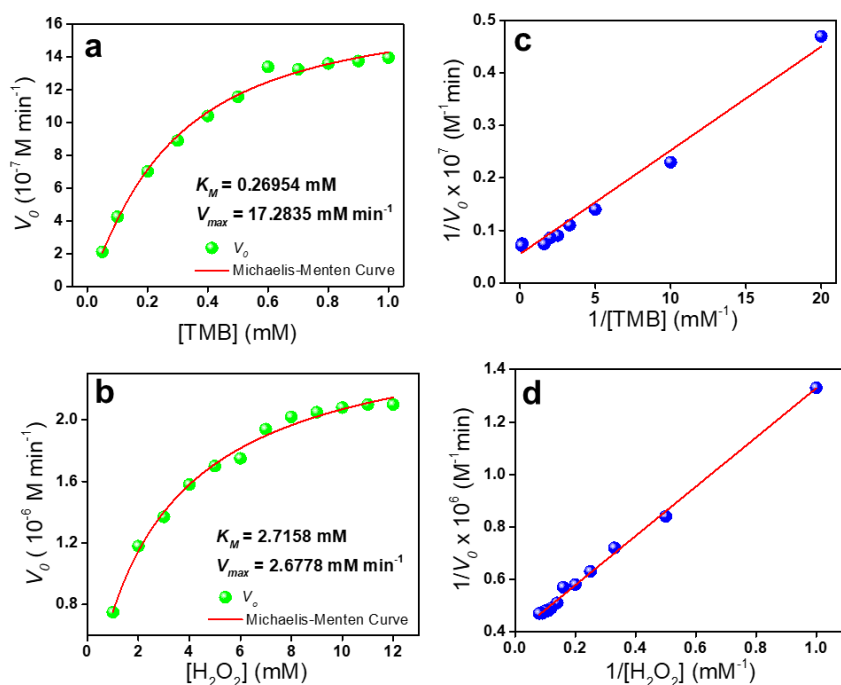


Figure 23. Michaelis-Menten-like enzymatic curves of the ADP-GMP hydrogel with respect to **(a)** TMB concentration (0.05-1.0 mM) and **(b)** H_2O_2 concentration (0.0-12 mM). Lineweaver-Burk plot of the ADP-GMP hydrogel with respect to the change in **(c)** TMB concentration and **(d)** H_2O_2 concentration.

The ADP-GMP hydrogel displayed a specific activity of 1.5 U/mg, highlighting its notable intrinsic catalytic efficiency and demonstrating that the assembled network effectively promotes peroxidase-like activity on a mass basis (Figure 24a). In addition, the ADP-GMP system retained robust peroxidase-mimetic performance over a broad temperature range of 15-30°C, with maximum activity observed at 30°C (Figure 24b). This combination of appreciable specific activity and temperature tolerance indicates that the hydrogel possesses good operational stability under mild and physiologically relevant conditions, supporting its potential utility in catalytic and biomedical applications. Notably, the ADG hydrogel exhibited a higher catalytic rate compared to the ATG system, which can be attributed to its more pronounced G-quadruplex stacking and superior structural organization. As the concentration of GMP dictates the extent of G-quadruplex (G4) formation, its effect on peroxidase-like activity was systematically evaluated by fabricating ADG hydrogels with varying GMP levels (Figure 24c). A progressive enhancement in catalytic velocity was observed with increasing GMP concentration, demonstrating a strong positive correlation between G4 formation and catalytic efficiency. This behavior highlights the crucial role of densely stacked G-quartet structures in modulating catalytic performance, where higher G4 content offers a greater number of accessible and efficient catalytic sites within the hydrogel network.

Furthermore, the enhanced catalytic activity at higher GMP concentrations may also arise from improved electron transfer pathways and increased local substrate concentration within the well-organized fibrillar network. The hierarchical assembly of G4 structures likely facilitates cooperative interactions, thereby amplifying the overall catalytic response. These findings underscore the importance of structural ordering and supramolecular density in designing G4-based catalytic hydrogels with tunable and enhanced enzymatic mimicry.

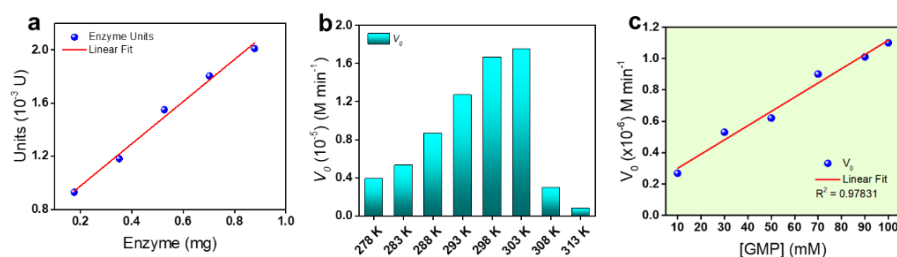


Figure 24. (a) Specific activity curve when TMB is used as substrate. (b) Effect of temperature on the peroxidase activity. (c) Concentration-dependent enhancement in catalytic velocity with increasing GMP content in the ADG hydrogel, highlighting the critical role of G-quadruplex (G-quartet) structures in governing catalytic activity.

Peroxidase-like catalytic systems are generally understood to proceed via two principal mechanisms: (i) a radical-mediated pathway involving the generation of highly reactive hydroxyl radicals ($\bullet\text{OH}$), and (ii) a direct electron transfer pathway. To elucidate the dominant mechanism in the present system, both electron paramagnetic resonance (EPR) spectroscopy and a terephthalic acid (TA) fluorescence assay were employed.

EPR spectroscopy is a powerful technique used to detect short-lived paramagnetic species, such as free radicals, by monitoring their unpaired electrons. Since hydroxyl radicals are extremely transient, a spin-trapping agent, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), was used to stabilize them. DMPO reacts with $\bullet\text{OH}$ radicals to form a more stable DMPO–OH adduct, which can be detected by EPR (Figure 25a). In both ADG and ATG systems, a characteristic quartet EPR signal with an intensity ratio of 1:2:2:1 was observed, which is a signature of the DMPO–OH adduct (Figure 25b). This result provides direct evidence for the generation of hydroxyl radicals, confirming that a radical-mediated pathway is operative in the catalytic process.

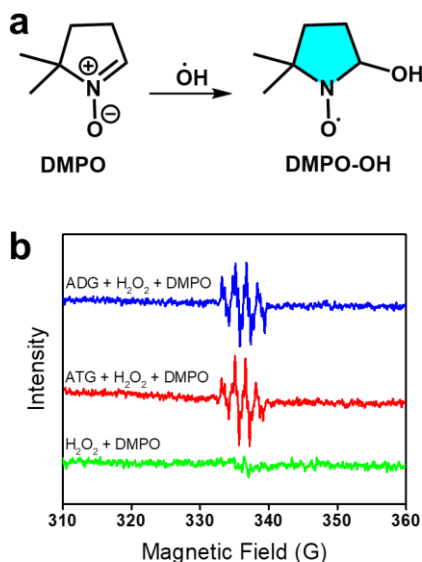


Figure 25: (a) Schematic reaction showing 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin-trapping agent. (b) Electron paramagnetic resonance (EPR) spectra obtained, showing a characteristic four-line signal corresponding to the DMPO- $\cdot\text{OH}$ adduct in the presence of ADG and ATG systems.

To further validate and quantify $\cdot\text{OH}$ generation, a terephthalic acid (TA) assay was performed. Terephthalic acid, a weakly fluorescent probe, was employed because it reacts with $\cdot\text{OH}$ radicals to yield the strongly fluorescent product 2-hydroxyterephthalic acid (Figure 26a). A marked increase in fluorescence intensity was observed in the presence of H₂O₂ and ADP-GMP condensates, whereas no significant fluorescence enhancement was detected in the absence of the catalyst. These results indicate that the ADP-GMP condensates promote the generation of $\cdot\text{OH}$ radicals during the peroxidase-like catalytic process (Figure 27b)^{41,42}. To further substantiate this observation, a time-dependent kinetic study was performed by monitoring the fluorescence intensity of the terephthalic acid reaction system at 5 min intervals over 30 min. A progressive increase in fluorescence intensity was observed with increasing reaction time, consistent with the continuous formation of 2-hydroxyterephthalic acid (Figure 27c). This time-dependent fluorescence enhancement further confirms the generation of $\cdot\text{OH}$ radicals in the presence of the ADP-GMP condensates.

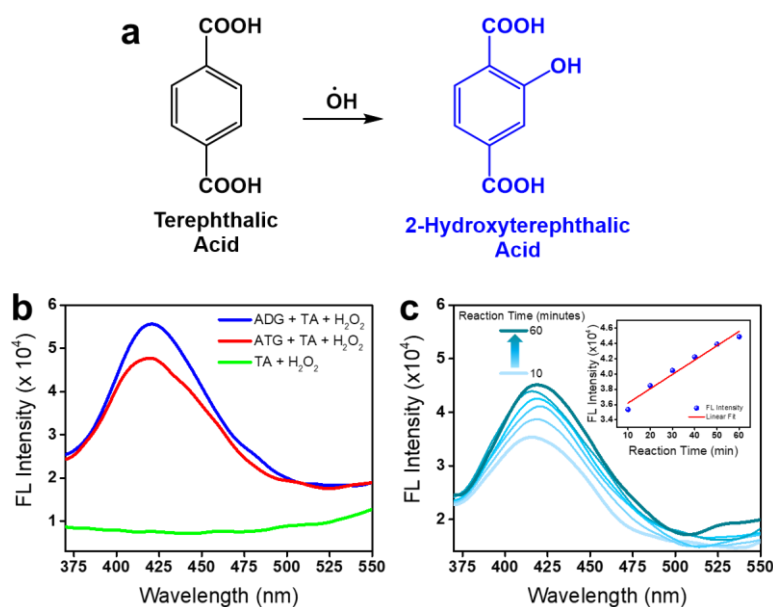


Figure 26. Fluorescence spectra of $\cdot\text{OH}$ generated in the reaction system: **(a)** Schematic illustration of the conversion of terephthalic acid to fluorescent 2-hydroxyterephthalic acid upon reaction with hydroxyl radicals ($\cdot\text{OH}$). **(b)** in the presence of the catalyst or in the absence of the catalyst. **(c)** Time-dependent Fluorescence of terephthalic acid.

6.13. Enabling Biocatalytic Cascade with “All-Nucleotide” Hydrogel

The hydrated and spatially confined architecture of the ADG hydrogel offers a highly suitable platform for enzyme immobilization and multi-step biocatalysis, leveraging its inherent peroxidase-mimicking activity. This hydrogel is formed through the co-assembly of nucleotide building blocks under aqueous conditions, resulting in a minimal yet functionally versatile matrix capable of co-encapsulating multiple enzymes while preserving their native structure and activity. The confined network promotes close spatial proximity between catalytic components, thereby enhancing substrate channeling and improving the efficiency of sequential reactions. Notably, the ADG hydrogel exhibits dual functionality: it serves both as a biomimetic microenvironment for enzyme encapsulation and as an active catalytic scaffold with intrinsic horseradish peroxidase (HRP)-like behavior. This combination of structural confinement and catalytic activity creates a

synergistic system that significantly improves cascade performance compared to conventional platforms. To demonstrate this concept, β -galactosidase (β -Gal) and glucose oxidase (GOx) were co-encapsulated within the hydrogel, enabling integrated enzyme–material cascade catalysis (Figure 27).



Figure 27. Schematic illustration of enzyme encapsulation (β -galactosidase and glucose oxidase) within the ADG hydrogel, highlighting its dual role as a confined catalytic milieu and intrinsic HRP-like scaffold.

In the two-component cascade system, glucose oxidase (GOx) catalyzes the oxidation of glucose to gluconic acid, simultaneously generating hydrogen peroxide (H_2O_2) as a byproduct. The in situ generated H_2O_2 is subsequently utilized by the ADG hydrogel, which exhibits intrinsic peroxidase-like activity, to oxidize the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Figure 28a). This reaction produces oxidized TMB (ox-TMB), characterized by a distinct blue color with an absorbance maximum at 652 nm.

Kinetic analysis of this system revealed a substrate concentration-dependent increase in absorbance, indicating enhanced catalytic turnover with increasing glucose concentration. The response remained linear within the investigated range, enabling reliable quantitative analysis. The limit of detection (LOD) for glucose was determined to be 22 μM . The high efficiency of this cascade can be attributed to the confined hydrogel environment, which minimizes diffusion limitations and facilitates rapid transfer of H_2O_2 between GOx and the hydrogel catalytic sites (Figure 28c).

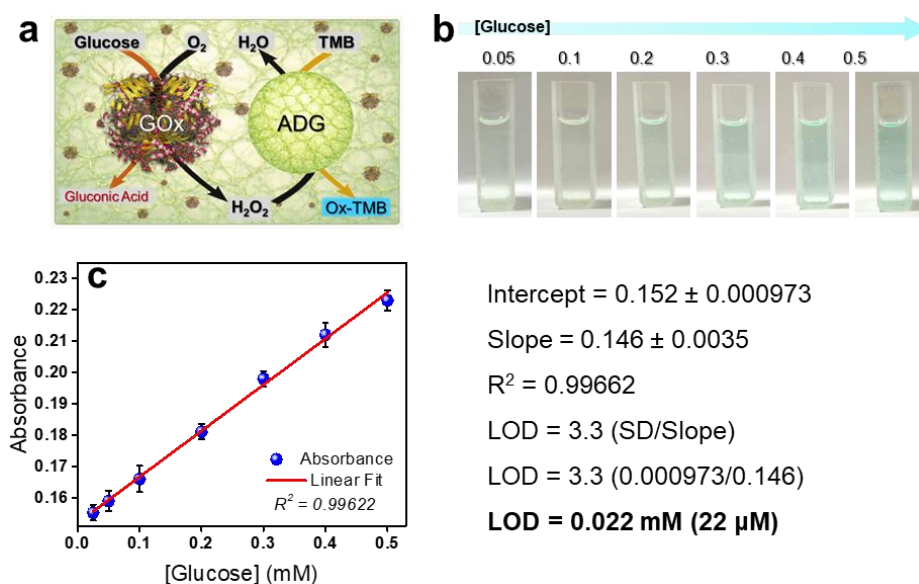


Figure 28. (a) A two-enzyme cascade system in which GOx catalyzes the oxidation of glucose to produce H₂O₂, which is subsequently utilized by the hydrogel to oxidize TMB into ox-TMB. (b) Photographic images showing concentration-dependent colorimetric response for glucose for the 2-step. (c) Calibration curves illustrating the linear correlation between absorbance and glucose concentration at a fixed reaction time (30 min) and constant TMB concentration (1 mM) for the cascade systems, along with corresponding limits of detection (LOD).

To further extend the cascade complexity, a three-component system was designed using lactose as the initial substrate. In this case, β -galactosidase (β -Gal) first hydrolyzes lactose into glucose and galactose. The generated glucose then enters the second step, where GOx catalyzes its oxidation to produce H₂O₂. Finally, the ADG hydrogel utilizes the generated H₂O₂ to oxidize TMB into ox-TMB, completing the cascade sequence (Figure 29a).

Although the three-step cascade involves an additional enzymatic reaction, resulting in slightly reduced overall reaction rates compared to the two-step system, it still exhibits a strong linear relationship between substrate concentration and absorbance. The limit of detection for lactose was calculated to be 23 μ M (Figure 29c). The marginal decrease in catalytic efficiency can be attributed to the

additional kinetic barrier associated with lactose hydrolysis. Nevertheless, the effective coupling between β -Gal, GOx, and the hydrogel catalytic network demonstrates efficient intermediate transfer and coordinated multi-step catalysis within the confined matrix.

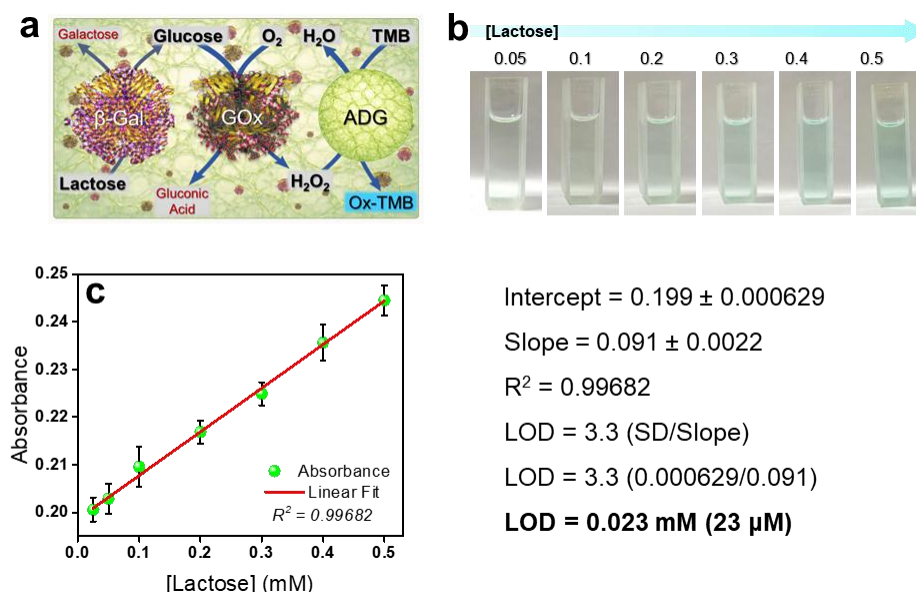


Figure 29. (a) Three-enzyme cascade system involving β -Gal-mediated hydrolysis of lactose to glucose, followed by GOx-generated H_2O_2 and hydrogel-catalysed TMB oxidation. (b) Photographic images showing concentration-dependent colorimetric response for lactose for the 3-step. (c) Calibration curves illustrating the linear correlation between absorbance and lactose concentration at a fixed reaction time (30 min) and constant TMB concentration (1 mM) for the cascade systems, along with corresponding limits of detection (LOD).

In both cascade systems, the progressive increase in absorbance at 652 nm, along with the visible intensification of the blue coloration of ox-TMB, confirms efficient catalytic activity and successful integration of enzymatic and material-driven processes. The linear dependence of absorbance on substrate concentration highlights the system's potential for quantitative sensing applications.

Overall, the enhanced catalytic performance arises from the unique properties of the ADG hydrogel, including reduced diffusion distances, improved intermediate transfer (particularly H_2O_2), and the synergistic

interplay between enzyme activity and intrinsic peroxidase-like functionality. These findings establish the ADG hydrogel as an effective catalytic microreactor capable of supporting complex cascade reactions while enabling sensitive and reliable substrate detection.

Chapter 7

Conclusion

In conclusion, this work demonstrates a simple yet powerful strategy for constructing fully nucleotide-based supramolecular hydrogels through the non-covalent condensation of GMP with adenosine phosphate derivatives. The study reveals that GMP acts as the primary structural unit, forming ordered G-quadruplex architectures via hydrogen bonding and π - π stacking, while ADP and ATP play a crucial role in modulating the physicochemical environment required for efficient assembly. The condensation process follows a hierarchical pathway involving liquid-liquid phase separation, droplet formation, and subsequent evolution into fibrillar networks, ultimately leading to hydrogel formation at higher concentrations.

A clear distinction in assembly behaviour was observed among the systems, where ADG and ATG exhibited robust fibrillation and gelation, whereas AMG failed to form stable networks. Among them, the ADG system showed superior structural organization, stability, and optimal conditions for higher-order assembly, highlighting the importance of balanced multivalent interactions and pH regulation.

Importantly, beyond structural assembly, the ADG hydrogel exhibits intrinsic peroxidase-like catalytic activity and functions as an efficient confined microreactor. The ability to support enzyme immobilization and facilitate multi-enzyme cascade reactions demonstrates its potential to mimic biological compartmentalization and enhance catalytic efficiency through substrate channelling and reduced diffusion limitations.

Overall, this study establishes a minimal and biogenic platform where self-assembly, structural organization, and catalytic functionality are intrinsically integrated within an all-nucleotide system. It provides significant insights into nucleotide-driven condensation mechanisms

and offers a versatile foundation for designing biomimetic materials with applications in catalysis, biosensing, and synthetic biology.

Chapter 8

Future Scope

The developed platform can be extended to complex multi-enzyme cascade systems, enabling efficient and selective artificial metabolic networks. Incorporation of feedback mechanisms may further allow the design of programmable biochemical reactors.

Introducing stimuli-responsive elements (pH, temperature, light, or enzymes) can provide dynamic control over assembly and catalysis, leading to adaptive, cell-like biomaterials. These hydrogels show strong potential in biosensing, drug delivery, and therapeutic catalysis due to their biocompatibility, tuneable microenvironment, and intrinsic activity under mild conditions.

Future efforts should focus on optimizing molecular composition and assembly parameters to enhance mechanical strength, stability, and catalytic efficiency, as well as integrating hybrid materials to expand functionality. Additionally, such systems offer a promising model for studying primitive biochemical processes relevant to prebiotic chemistry. Overall, this work paves the way for next-generation biomolecular materials with integrated structure and function.

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