

Exploring Nucleotides as Low Molecular Weight Gelators to Fabricate Supramolecular Multifunctional Hydrogels for Versatile Applications

Ph.D. Thesis

By

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**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY INDORE
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Exploring Nucleotides as Low Molecular Weight Gelators to Fabricate Supramolecular Multifunctional Hydrogels for Versatile Applications

A THESIS

*Submitted in partial fulfillment of the
requirements for the award of the degree
of*
DOCTOR OF PHILOSOPHY

by
VIDHI AGARWAL



**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY INDORE
JULY 2025**



INDIAN INSTITUTE OF TECHNOLOGY INDORE

I hereby certify that the work which is being presented in the thesis entitled **Exploring Nucleotides as Low Molecular Weight Gelators to Fabricate Supramolecular Multifunctional Hydrogels for Versatile Applications** in the partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** 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 **JANUARY 2020** to **JULY 2025** under the supervision of **Dr. TRIDIB KUMAR SARMA**, Associate Professor, Department of Chemistry, Indian Institute of Technology 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.

VIDHI AGARWAL

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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VIDHI AGARWAL has successfully given his/her Ph.D. Oral Examination held on 27.02.2026

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Dr. TRIDIB KUMAR SARMA

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*Dedicated to My
Family, Friends, and
Teachers*

SYNOPSIS

1. Introduction

Molecular self-assembly is ubiquitous in nature and can be defined as the spontaneous organization of molecules to form higher-order architectures. It is facilitated by several non-covalent interactions such as electrostatic interactions, hydrogen bonding, π - π stacking, and hydrophobic interactions [1-3]. Self-assembly is governed by the principles of supramolecular chemistry, elucidating the instructions for constructing superstructures encoded within the structural motifs of individual building blocks. Gelation represents a sophisticated form of higher-order self-assembly, wherein a precise orchestration of non-covalent interactions drives the hierarchical organization of molecular building blocks to form extended supramolecular networks. The resulting three-dimensional architectures facilitate the confinement of water within their interconnected fibrillar framework. Based on the nature of the solvent immobilized within the self-assembled network, gels are broadly classified into two categories: Organogels are formed when an organic solvent constitutes the continuous phase. Organogels are utilized in diverse applications such as oil spill recovery, organic solvent removal, and cosmetics. In contrast, hydrogels can trap or encapsulate water inside the matrix, rendering them highly relevant for biomedical and other aqueous-based technologies [4]. Hydrogels represent a class of material inherently aligned with the biological tissues due to their high-water content, flexibility, and elastic nature [5]. Consequently, the design of multifunctional smart materials leveraging the unique properties of hydrogels has emerged as a critical focus in advanced material research.

The hydrogel landscape is predominantly governed by polymeric gelators, but they have certain limitations such as limited water solubility, batch-to-batch variations, suboptimal biocompatibility, and

biodegradability, limiting their practical implications. In this context, small biomolecule low molecular weight gelators (LMWG) such as peptides, amino acids, and other organic molecules are being explored. [6,7]. Among all biomolecules, purine nucleotides are an ideal candidate as LMWG owing to their unique planar, heteroatom-rich, aromatic structures, providing multiple sites for hydrogen bonding and other non-covalent interactions [8,9]. Moreover, the presence of the phosphate group in their structure facilitates metal coordination. All these intriguing features collectively render them an ideal low molecular weight gelator. Multifunctional hydrogels can be rationally designed through the strategic selection of precursors to meet specific application requirements. Furthermore, their stimuli responsiveness, injectability, self-healing, and shape-sustaining behaviour add to their applicability.

In this thesis, we focused on the design and development of multifunctional hydrogels using nucleotide as the low molecular weight gelator. The fabrication of hydrogel through a simple, efficient, and facile process involving the use of pristine nucleotides as gelators highlights a distinct advantage of this approach in the development of functional soft materials. The pristine form of precursors is preserved in the hydrogel, thereby inheriting their intrinsic properties in the functional material. The structural, morphological, and mechanical aspects of the synthesized hydrogels are investigated using various spectroscopic and microscopic techniques. Finally, the hydrogels were exploited for diverse applications such as enzyme mimetics, energy generation, and other biomedical applications.

2. Summary of the Thesis

The thesis is structured into 5 chapters, and a brief overview of each chapter is described below.

2.1. Chapter 1. General Introduction and Background

This chapter provides an overview of supramolecular self-assembled gels, including their classification and the fundamental principles governing their formation. The importance and understanding of low

molecular weight gelators are being discussed with a primary focus on nucleotide as a biomolecule-based LMWG. This chapter also discusses recent advancements and emerging applications across diverse scientific domains, underscoring the growing relevance of this field.

2.2 Chapter 2. Synthesis and Characterization of Cobalt Adenosine Monophosphate (Co-AMP) Hydrogel

This chapter discusses the synthetic methodology and detailed characterization of the cobalt adenosine monophosphate hydrogels. Two sets of hydrogels are prepared at the Co^{2+} : AMP ratio of 1:1 and 1:2. The chapter comprises the concentration optimization, thermal, and pH stability of hydrogels. The spectroscopic, microscopic, and rheological characterization of both the hydrogels. Interestingly, the hydrogel formation is accompanied by a distinct visual color change from pink to blue, indicative of an underlying structural transition. Spectroscopic analysis confirmed the change in arrangement of the cobalt centre from an octahedral to a tetrahedral environment. The rheological analysis revealed the mechanical stability of this hydrogel. The broad functional potential of this hydrogel is demonstrated through its integration for a variety of applications, which is discussed in the subsequent chapter.

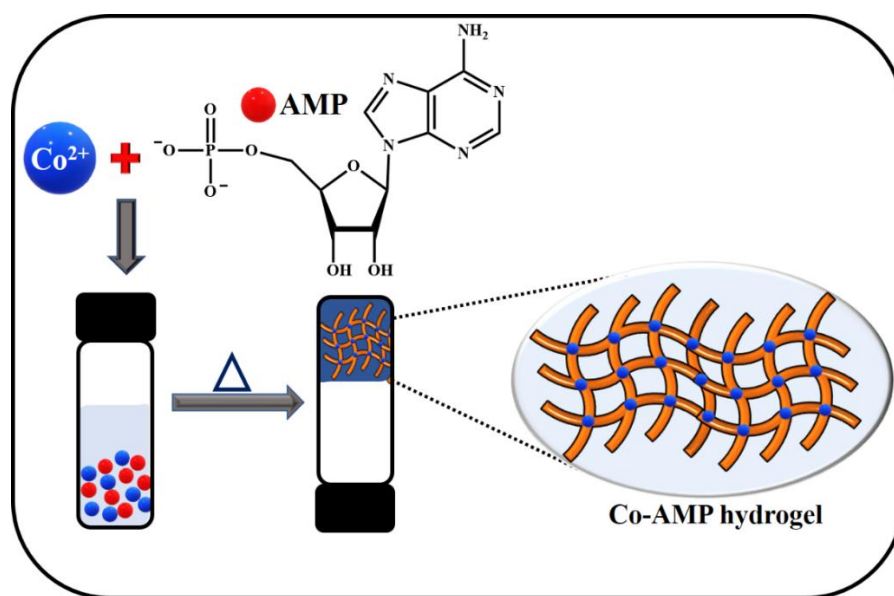


Figure 1. Synthetic scheme for the fabrication of Co-AMP hydrogel.

2.3 Chapter 3. Multifunctional Co-AMP Hydrogel for Versatile Applications

This chapter highlights the multifaceted applications of Co-AMP hydrogel and is divided into three sections, where each section includes the specific application of Co-AMP hydrogel. This hydrogel demonstrates significant potential in areas such as enzyme mimetics and electrochemical water splitting. The concise overview of specific application is discussed in the chapters discussed below:

2.4 Chapter 3A. Cobalt-adenosine monophosphate (Co-AMP) Supramolecular Hydrogel with pH-responsive Multi-Nanozymatic Activity

In this chapter, we discuss the multienzyme mimicking behaviour of Co-AMP hydrogel exhibiting pH-responsive catalase and peroxidase activity. Interestingly, the hydrogel displays catalase-mimicking activity under neutral and basic conditions, whereas in acidic media, it exhibits peroxidase-like behavior. The multifunctionality of the synthesized hydrogel was further demonstrated by phenoxazinone synthase-like activities. Owing to its catalase-mimicking activity, the hydrogel could effectively reduce the oxidative stress produced in cells due to excess hydrogen peroxide by degrading H_2O_2 to O_2 and H_2O under physiological conditions. The biocompatible hydrogel could prevent cell apoptosis by scavenging reactive oxygen species (ROS). A green and simple synthetic strategy utilizing commonly available biomolecules makes this hydrogel highly attractive for catalytic and biomedical applications.

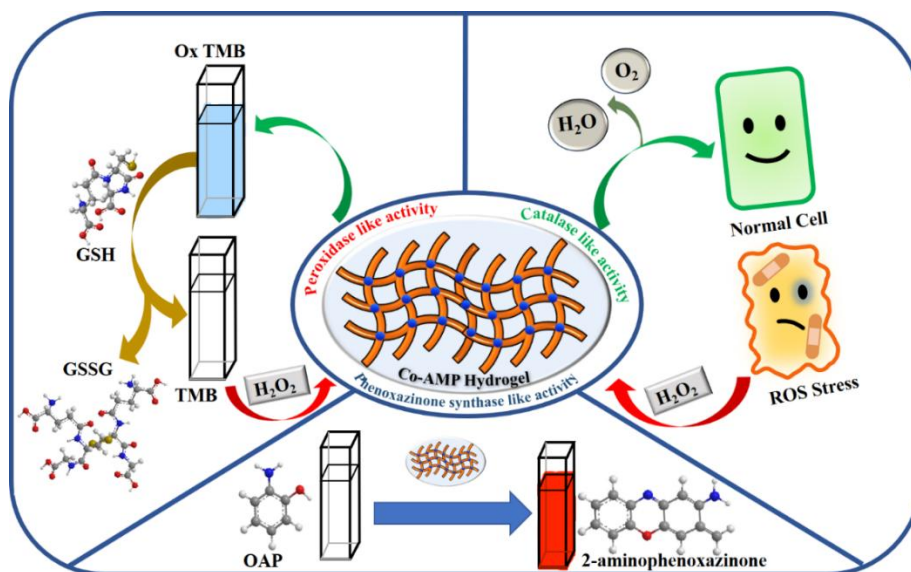


Figure 2. Schematic representation of the multienzyme activities of the Co-AMP hydrogel. The catalase mimicking activity was utilized to reduce the oxidative stress in the cell.

2.5 Chapter 3B. Supramolecular Cobalt-Nucleotide Hydrogel as a Non-Zinc and Non-Histidine Carbonic Anhydrase Mimic.

The alarming and sustained increase in atmospheric CO₂ levels presents a critical environmental challenge, contributing significantly to global warming and the associated climate crisis. In response, substantial research efforts are directed toward developing efficient and sustainable strategies for CO₂ capture and fixation. Nature addresses this issue with the help of the carbonic anhydrase (CA) enzyme, which catalyzes the hydration of CO₂ to form bicarbonate. Carbonic anhydrase contains zinc coordinated to three histidine residues and one water molecule in a tetrahedral manner. However, the economic and other operational aspects limit the practical implications of natural enzymes. As a result, the development of robust and economical enzyme mimics has emerged as a promising alternative. In this context, this chapter highlights the exploitation of Co-AMP hydrogel as a functional mimic of carbonic anhydrase. Interestingly, this is the first report where a non-zinc and non-histidine CA mimic has been developed. It exhibited CA-like biocatalytic activity towards both ester hydrolysis and hydration of CO₂ in aqueous medium. This chapter includes the enzymatic assays and

the calculation of kinetic parameters in detail. The CA mimicking activity of this hydrogel is also utilized for the biomineralization of heavy toxic metals like Cd and Pb. Also, the biomineralization of Ca led to the formation of carbonated hydroxyapatite, which is close to the composition of the bone. It can be employed in bone regeneration, leveraging better biosorption than pure hydroxyapatite. The facile synthetic methodology and the stability of this hydrogel across a wide range of pH and temperature make it suitable for practical deployment.

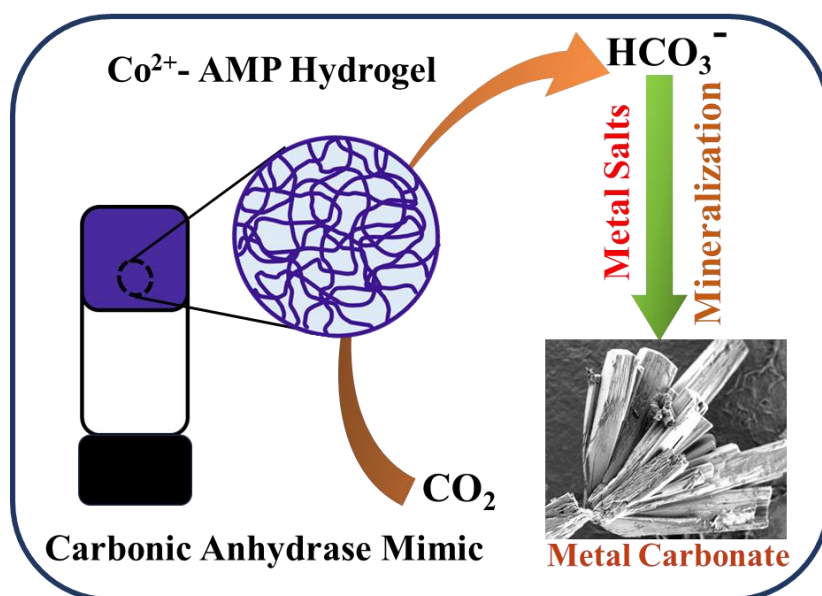


Figure 3. Schematic representation of Co-AMP hydrogel as a functional mimic of carbonic anhydrase.

2.6 Chapter 3C. A Tetrahedral Coordinated Cobalt-Nucleotide Hydrogel for Electrocatalytic Overall Water Splitting

Amid the global drive to eliminate carbon emissions and mitigate the energy crisis, water splitting emerges as a beacon of innovation, converting the most abundant resource on earth into clean hydrogen. The pursuit of efficient, cost-effective, and earth-abundant metal-based electrocatalysts with facile synthesis and reproducibility still rages on. This chapter highlights the utilization of a cobalt-adenosine monophosphate (Co-AMP) hydrogel as a bifunctional catalyst for overall water splitting in alkaline conditions with an overall cell voltage of 1.56 V at 10 mA cm⁻², outperforming the benchmark

catalytic system $\text{IrO}_2\|\text{Pt/C}$ (1.62 V at 10 mA cm^{-2}). Three crucial factors have played important roles in the elegant performance of this hydrogel system for water splitting: (1) The PO_4^{3-} ions in the AMP, which are coordinated with the central Co^{2+} ions facilitate efficient adsorption of key intermediates involved in the reaction, (2) the tetrahedral arrangement of the nucleotides around the cobalt atom, makes the active site more exposed and accessible, enabling better interaction with reactants and intermediates, (3) The formation of hydrogel enhances electrolyte confinement resulting in faster reaction kinetics. The integration of all stringent features enhanced its efficiency and applicability. Interestingly, at $70\text{ }^\circ\text{C}$, the hydrogel achieves an exceptionally low cell voltage of 1.42 V at 1 M KOH , setting a new benchmark for non-noble metal electrocatalysts. Under harsh corrosive conditions ($30\text{ wt\% KOH}$) the hydrogel requires just 1.60 V to achieve a current density of 10 mA cm^{-2} , underscoring its durability and industrial scalability. This hydrogel exemplifies the optimal convergence of critical parameters and offers a guiding framework for the rational design of efficient and advanced bifunctional catalysts.

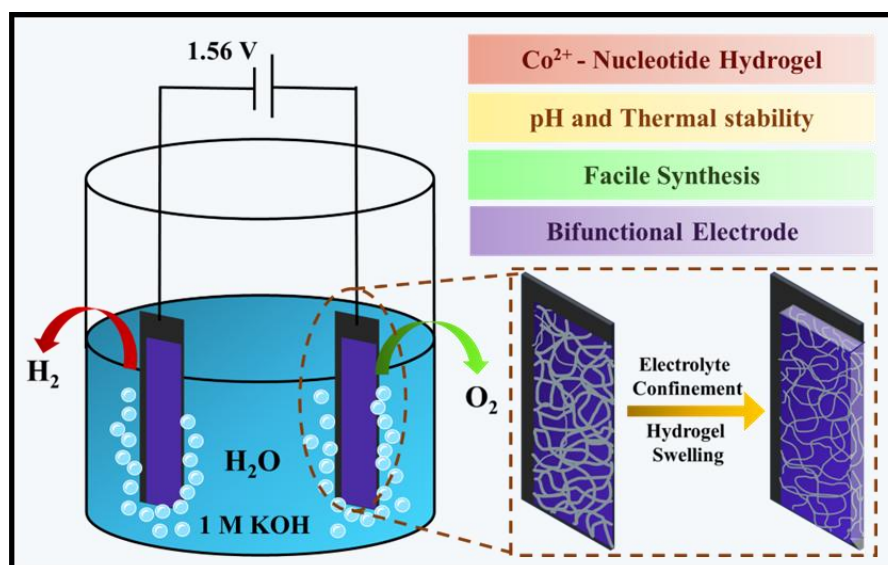


Figure 4. Schematic representation of Co-AMP hydrogel as a bifunctional electrocatalyst for electrochemical overall water splitting in an alkaline medium.

2.7 Chapter 4. Glutathione-Nucleotide Supramolecular Hydrogels with Intrinsic Antiviral, Antibacterial, and Anticancer Activities

In Chapter 4, we discussed a facile methodology to fabricate two glutathione-based hydrogels utilizing guanosine monophosphate (GMP) as a gelator. The simple physical mixing of two components resulted in a spontaneous, transparent, and mechanically robust hydrogel. The hydrogels are stable in a wide pH window and show thermo-reversibility, self-healing, thixotropic, and injectable behaviour. The concentration-dependent morphological studies revealed that the formation of helical fibrils takes place via protocell-like spherical intermediates. The all-biomolecule hydrogel is biocompatible, and the high water content in the matrix enables it to mimic a tissue-like environment, further augmenting its suitability for biological applications. This chapter also reflects the importance of preserving the native form of biomolecules in the functional material. It also discusses the *in vitro* cell studies conducted to explore the multimodal bioactivities of the hydrogels. The results revealed the potent antiviral activity of the hydrogels against EBV and SARS-CoV-2 viruses. These hydrogels also possess antibacterial activity against both gram-positive and gram-negative bacteria. qRT-PCR, cell proliferation, and EB/AO staining experiments revealed the induced cell apoptosis in gastric cancer cells upon hydrogel treatment. The multimodal bioactivities of the hydrogels can be exploited to design materials for inhibiting microbial propagation and facilitating therapeutic applications to tackle COVID-19-like outbreak situations and minimize health hazards. The viscoelastic and shape-sustaining behaviour of these hydrogels can be potentially utilized to develop antimicrobial coatings on various surfaces.

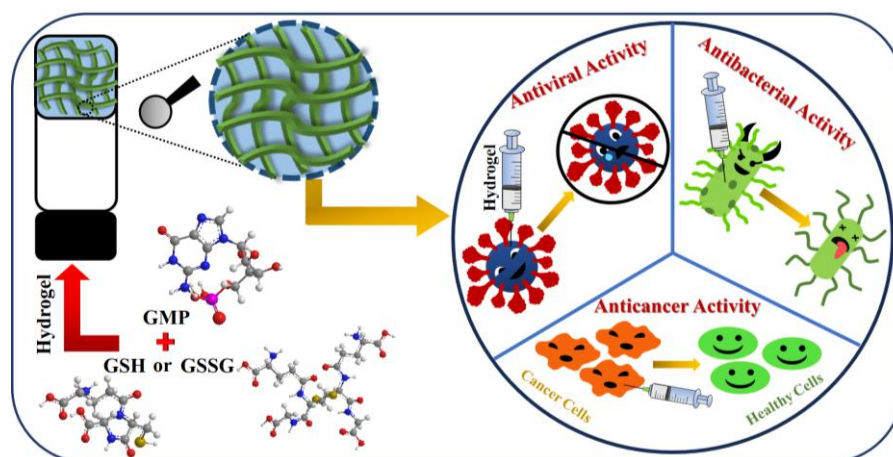


Figure 5. The schematic representation for the synthesis and multimodal bioactivities of the Glutathione-GMP hydrogels.

2.8 Chapter 5. Conclusion and Future Outlook

The last chapter highlights the importance of the research work presented in the thesis and delineates its prospective impact and relevance in future scientific advancements. This thesis primarily focuses on the design and development of supramolecular hydrogels using nucleotides as the low molecular weight gelator. The proposed facile synthetic methodology, which is devoid of any chemical modification, is one of the unique aspects, thereby preserving the native structure and functionality of the biomolecules in the functional material. Also, the utilization of nucleotides as LMWG addresses the biocompatibility concerns and widens their utility for biomedical applications. The hydrogels were explored for diverse applications such as enzyme mimetics, energy generation, and other biomedical applications, highlighting the potential of these soft materials as multifunctional platforms in advanced material science. Furthermore, the strategic selection of crosslinking moieties enables to design a variety of hydrogel systems, thereby expanding their applicability across a broad spectrum of functional domains for different applications.

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LIST OF PUBLICATIONS

Thesis Work:

1. **Vidhi Agarwal**, Nidhi Varshney, Surbhi Singh, Nitin Kumar, Amrita Chakraborty, Bhagwati Sharma, Hem C Jha*, Tridib K. Sarma*. Cobalt-Adenosine Monophosphate (Co-AMP) Supramolecular Hydrogel with pH-responsive Multi Nanozymatic Activity, *ACS Appl. Bio Mater.* **2023**, 6, 5018-5029. (DOI: 10.1021/acsabm.3c00719)
2. **Vidhi Agarwal**, Vaishali Saini, Aditya Prasun, Nidhi Varshney, Amrita Chakraborty, Hem Chandra Jha*, Tridib K. Sarma*. Peptide-nucleotide supramolecular hydrogel with intrinsic antibacterial, antiviral, and anticancer activities. (*Manuscript Under Revision*)
3. **Vidhi Agarwal**, Bidyut Chutia, Ravindra Vishwakarma, Aditya Prasun, Amrita Chakraborty, Sasanka Deka*, Tridib K. Sarma*. Tetrahedral coordinated cobalt-adenosine monophosphate heat-set hydrogel for electrochemical overall water splitting. (*Manuscript Submitted*)
4. **Vidhi Agarwal**, Tridib K. Sarma*. Non-zinc histidine cobalt based supramolecular hydrogel as carbonic anhydrase mimic. (*Manuscript Under Preparation*)

Other Publications:

1. Shivendra Singh[‡], **Vidhi Agarwal[‡]**, Tridib K Sarma*, Tushar Kanti Mukherjee*. Metal-free photocatalysis at charged aqueous interfaces: boosting the photocatalytic oxidative coupling of arylamines to azoaromatics under ambient conditions. *Green Chem.*, 2023, 29, 9109-9114. (‡ = **Equal Contribution**) (DOI:10.1039/D3GC03150K)
2. **Vidhi Agarwal[‡]**, Amrita Chakraborty[‡], Aditya Prasun[‡], Tarun Kumar Sahu[‡], Tridib K Sarma*. Carbon Dots as Versatile Metal-

Free Carbocatalysts for Organic Transformations, *ChemCatChem* (DOI:10.1002/cctc.202500414)

3. Tarun Kumar Sahu, Ankit Gupta, **Vidhi Agarwal**, Tridib K. Sarma*. Facile Synthesis of Enzyme-Mimicking Cobalt-Polyphenol Coordination Network for Oxidation of Phenolic Pollutants. *ACS Appl. Eng. Mater.* **2023**, *12*, 3194–3204. (DOI:10.1021/acsaenm.3c00487)
4. Amrita Chakraborty, Saswati Dash, Neha Thakur, **Vidhi Agarwal**, Debasis Nayak*, and Tridib K. Sarma*. Polyoxometalate-Guanosine Monophosphate Hydrogels with Haloperoxidase-like Activity for Antibacterial Performance. *Biomacromolecules* **2024**, *25*, 104–118. (DOI:10.1021/acs.biomac.3c00845)
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6. Amrita Chakraborty, Tanushree Ghosh, Suryakamal Sarma, **Vidhi Agarwal**, Rajesh Kumar*, Tridib K. Sarma*. A multi-stimuli responsive polyoxometalate-guanosine monophosphate hybrid chromogenic smart hydrogel. *J. Mater. Chem. C* **2024**, *12*, 13447-13456. (DOI: 10.1039/D4TC01973C)
7. Amrita Chakraborty, Annu Rani, Pramesh Sinha, Suryakamal Sarma, **Vidhi Agarwal**, Aditya Prasun, Hem Chandra Jha*, Tridib K Sarma*. Guanosine Monophosphate Induced Solubilization of Folic Acid Leading to Hydrogel Formation for Targeted Delivery of Hydrophilic and Hydrophobic Drugs. *ACS Appl. Mater. Interfaces* **2025**, *17*, 11848–11860. (DOI: 10.1021/acsami.4c21306)
8. Neha Thakur, Suryakamal Sarma, **Vidhi Agarwal**, Amrita Chakraborty, Tridib K Sarma*. Acidic Amino Acid Induced 5'-Guanosine Monophosphate Fibrillar Nanozyme: A Highly

Efficient Metal-free Intrinsic Peroxidase Mimic. (Under Review)

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NOMENCLATURE

$\text{\AA}$ **Angstrom**

λ Wavelength

μ Micro

π Pi

mM Milli Molar

μM Micro Molar

cm Centimeter

μm Micrometer

nm Nanometer

$^{\circ}$ Degree Centigrade

K Kelvin

mL Millilitre

mV Milli Volt

V Volt

eV Electron Volt

μL Microlitre

a. u. Arbitrary Unit

λ_{ex} Excitation Wavelength

λ_{em} Emission Wavelength

pH The negative logarithm of
hydronium-ion concentration

M	Molar concentration
mg	Milligram
s	Seconds
Oe	Oersted

ACRONYMS

3D	Three dimensional
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
AMP	Adenosine monophosphate
GMP	Guanosine monophosphate
LMWG	Low molecular weight gelator
CPGs	Coordination polymer gels
GSH	Reduced glutathione
GSSG	Oxidized glutathione
MB	Methylene Blue
RhB	Rhodamine B
ThT	Thioflavin T
UV-Vis	Ultraviolet-visible
FTIR	Fourier Transform Infrared
NMR	Nuclear magnetic resonance
CD	Circular dichroism
AFM	Atomic Force Microscopy
CLSM	Confocal laser scanning microscopy
FESEM	Field emission scanning electron microscopy
TEM	Transmission electron microscopy
XPS	X-ray photoelectron microscopy

PXRD	Powder X-ray diffraction
EIS	Electrochemical impedance study
FL	Fluorescence
AS	Amplitude sweep
FS	Frequency sweep
FESEM	Field emission scanning electron microscopy
Co-AMP	Cobalt adenosine monophosphate
EDTA	Ethylenediaminetetraacetic acid
HRP	Horseradish peroxidase
CA	Carbonic anhydrase
LSV	Linear sweep voltammetry
TOF	Turnover frequency
OER	Oxygen evolution reaction
HER	Hydrogen evolution reaction
OWS	Overall water splitting
DFT	Density functional theory

Chapter 1

Introduction and General Background

1.1 Introduction

The study of chemical evolution and the origin of life represents a profoundly interdisciplinary field, encompassing all branches of natural sciences. Over the years, nature has strategically employed self-assembly to orchestrate the transformation of simple molecular precursors into structurally and functionally sophisticated architectures to operate in an integrated manner within living systems [1-4]. Supramolecular chemistry, also known as “chemistry beyond the molecule” focuses on the study of higher-order assemblies formed through non-covalent interactions. The field gained prominence following the landmark contributions of Donald J. Cram, Jean-Marie Lehn, and Charles J. Pedersen, who were jointly awarded the Nobel Prize in Chemistry for their pioneering work that fundamentally revolutionized the field [5,6]. Since then, supramolecular chemistry has evolved exponentially, expanding the horizon of supramolecular chemistry to the multimolecular systems. It now serves as a crucial interdisciplinary nexus, integrating principles from chemistry, biology, and physics to address challenges across diverse scientific domains [5-7]. Self-assembly stands as the driving force behind the dynamic complexity of supramolecular systems and is defined as the spontaneous molecular arrangement of disordered molecules into ordered structures facilitated by specific local interactions. It is a ubiquitous process in nature that underlies the formation of complex biological systems. Notably, both life and the human body in particular are sophisticated construct of nature, which represents the highest level of chemical self-assembly. Biomolecules like proteins, peptides, lipids, nucleic acids, and various other cellular components self-assemble to form the cell, the elementary biological unit. Some examples of naturally occurring self-assemblies include the lipid bilayer membrane of cells, chromatin

assembly, RNA, and DNA complexes [8-11]. These self-assembled structures play crucial roles in physiological functions and life activities, such as antigen-antibody recognition, amyloid fibril formation, and membrane formation [12,13]. These natural architectures always motivated the researchers to develop artificial materials with hierarchical structures and tailored properties for the fabrication of multifunctional and advanced biomaterials. Among these biomaterials, gels are emerging as versatile, intelligent materials integrating multiple scientific domains, and also share a key resemblance to biological tissues [14]. Representing a unique form of molecular self-assembly, gels form through the organization of small molecules into supramolecular three-dimensional networks. The upsurge in gel-focused research over the last two decades underscores the critical importance of developing novel hydrogel systems [15,16]. This approach will lead to the integration of molecular science and bioinformatics for the betterment of mankind.

1.2 Fundamentals of gels

Gels are soft materials with numerous roles in industries and are quite popular in everyday life. These are viscoelastic semi-solid materials comprising a three-dimensional crosslinked network that can entrap a large amount of solvent.

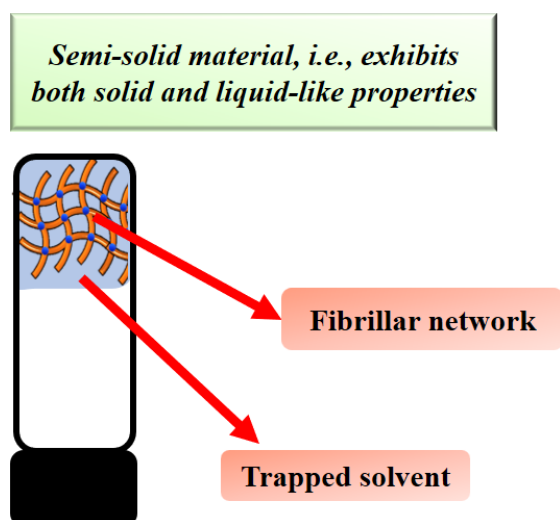


Figure 1.1 Pictorial representation of a gel showing the coexistence of two phases.

As per IUPAC, “Gels are defined as a nonfluid colloidal network or polymer network that is expanded throughout its whole volume by a fluid” [17-19]. The gels are archetypal nanomaterials in which information is transduced from the molecular state to the macroscopic state. The gel matrix arises due to the coexistence of two different phases: a ‘liquid-like’ phase constituted by the large amount of solvent and a solid-like phase (3D network) that restricts the flow of the liquid [20,21]. The formation of gel can be preliminarily ascertained with the ‘vial inversion test’, i.e., when a vial containing gel is inverted upside down, it does not flow under the influence of gravity, indicating the formation of a self-supporting three-dimensional network (**Figure 1.1**). The term “gel”, derived from gelatin, was first introduced by the Scottish chemist Thomas Graham in 1861 [22]. Decades later, in 1926, Dorothy Jordan Lloyd stated, “the gel is easier to recognize than to define”. Further, in 1974, Paul Flory provided a structural perspective, defining gels as materials with a continuous microstructure that retains solid-like behaviour up to a critical strain threshold, while exhibiting liquid-like flow after a certain strain value. The solid-like phase constitutes a very small percentage of the overall material, with the constituent molecules forming the network being known as gelators. Gelator is the basic unit of gel that can be chemically crosslinked through covalent bonds or physically linked to one another by non-covalent interactions [21,23,24]. Gels are versatile soft materials and owing to their unique features, they have found application in diverse fields of science, ranging from catalysis, energy conversion and storage, textiles, to biotechnology and other biomedical applications [25-31].

1.2.1 Classification of Gels

Gels can be categorized on the basis of several parameters, including the solvent medium or the liquid phase, the type of crosslinking of the 3D network (solid phase), and their composition (**Figure 1.2**). The gels can be classified into two categories on the basis of the solvent used. When the water serves as the solvent, the resulting

gel material is called a hydrogel, while if the organic solvent is used, it is defined as an organogel [32,33].

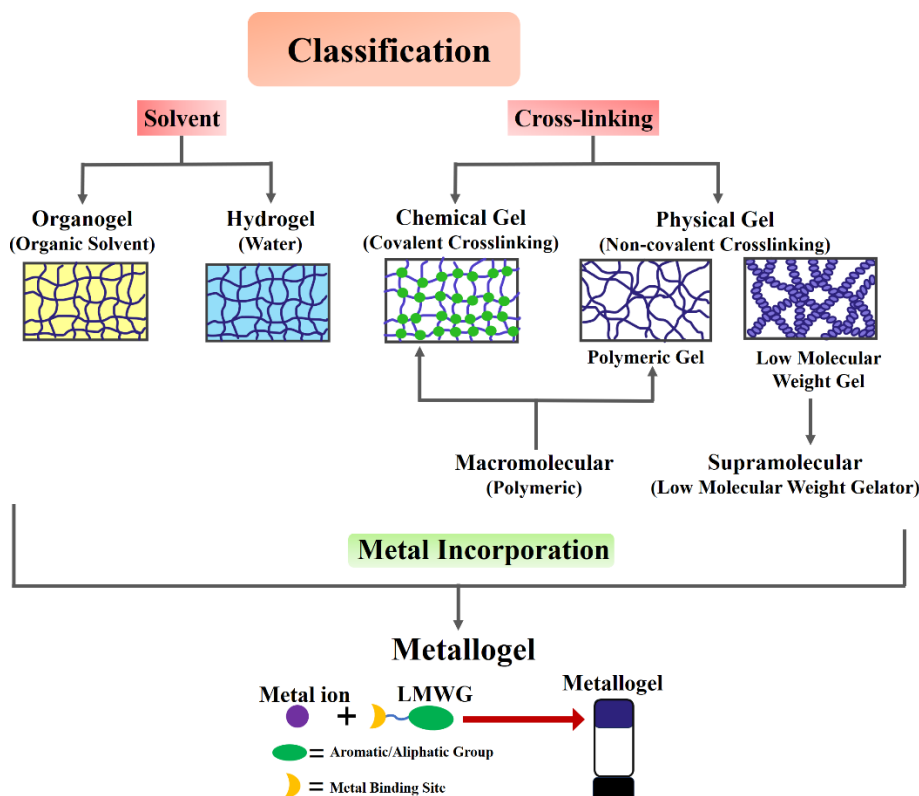


Figure 1.2 Pictorial representation showing the classification of gels.

Further, they have been classified based on their structural constituents. If the polymer is used for forming the 3D network, they are macromolecular gels, while the gels fabricated using low molecular weight gelators are termed as supramolecular gels [34]. The resultant material is a chemical gel if its 3D network is stabilized by permanent covalent crosslinking. In contrast, physical gels arise from reversible dynamic non-covalent interactions. Metallogels represent another distinct class of gels, characterized by the presence of metals within their matrix. Metallogels are further divided into two subcategories: the first comprises coordination polymer gels, where metal is the integral part of the crosslinked network, and the gelation is induced by metal-ligand coordination. On the contrary, in another case, the discrete metal complexes are self-assembled through non-covalent interactions. The metals are an integral part of the gelator but do not directly participate in the formation of the fibrous crosslinked network. The metallogels

provide an integrated platform where the properties of both metal ions and hydrogel counterpart can be exploited towards the development of multifunctional soft materials [35]. The detailed discussion regarding the metallogels will be presented in the subsequent section.

1.3 Importance of Hydrogels

Hydrogels are an integral entity of nature, finding their existence in almost every segment of life. Moreover, their hydrated matrix allows them to resemble a tissue-like structure. The extracellular matrix of the cells, the gelatinous fluid inside the cell, i.e., cytoplasm, vitreous humor in the eyes are all hydrogels [36,37]. Interestingly, Yuk et al. stated, “the human body is a hydrogel soft robot” [38]. In this regard, hydrogels are of significant importance for several biomedical applications, surpassing the organogels. Though organogels have their own set of benefits, such as the choice of the liquid phase is much wider, enabling diverse applications such as oil spill recovery, separation, organic solvent removal, ice-phobic surfaces, and cosmetic products [39-42]. Moreover, the thermal stability of an organogel can be modulated depending on the boiling point of the chosen solvent. But they have certain limitations, such as toxicity, hydrophobicity, and poor conductivity, that limit their utility in biomedical and other applications [43]. In this regard, hydrogels are emerging as a wonder material and covering a range of versatile applications such as drug delivery vehicles [44-46], bone regeneration [47-49], tissue engineering [50-52], contact lenses [53,54], wound healing [55-57], and immunotherapy [58,59]. Apart from biomedical applications, hydrogels are utilized in different domains of science like catalysis, energy storage and energy generation, electrocatalysis, etc [60-62]. Hence, it is very crucial to understand their molecular interactions and develop novel hydrogel systems with desired specific requirements.

The hydrogel landscape is predominantly governed by polymeric gelators such as gelatin, chitosan, agar, cellulose, as natural polymers, while polyvinyl alcohol, polyethylene glycol, and

polyacrylamide, etc. as synthetic polymers [63-65]. Certain limitations associated with polymeric hydrogels, like batch-to-batch synthetic variations, limited water solubility, suboptimal biocompatibility, and biodegradability, restrict their practical implications [66]. In this context, over the past two decades, the design and development of low molecular weight gelators (LMWGs) has garnered significant interest, driven by their ability to form gels guided by supramolecular protocols [21,23,67,68]. This class of hydrogels represents a compelling category of materials having intriguing features and multidimensional applications, as highlighted in the following sections (**Figure 1.3**) [25-69].

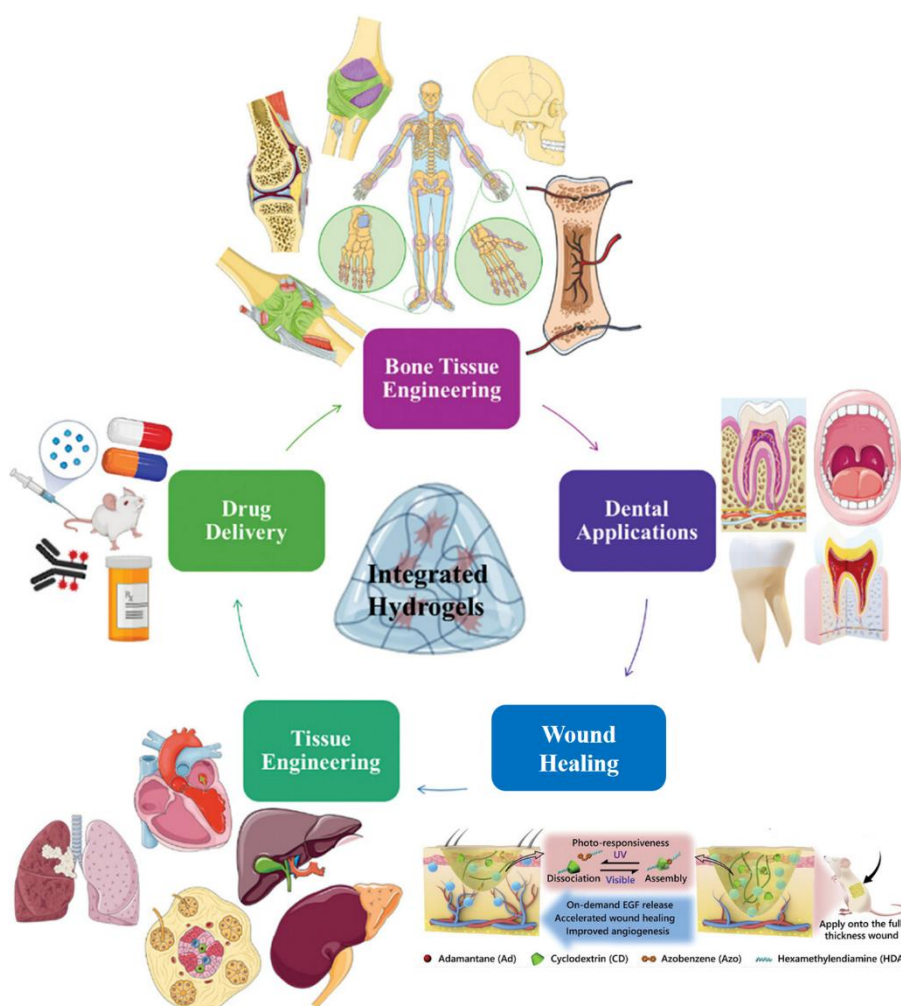


Figure 1.3 Biomedical applications of hydrogels. [Adapted from references 26, 69]

1.4 Low Molecular Weight Gelators (LMWGs)

As we discussed above, the gelator can be broadly classified into two categories: The polymeric gelator, where long chain polymer chains facilitate the gelation through covalent or non-covalent crosslinking. The other category of gelator is low molecular weight gelators that are arbitrarily outlined as having molecular weight lower than 3000 Da [70]. These small molecules induce gel formation, leveraging the non-covalent interactions [68]. A typical gelator is expected to exhibit certain fundamental structural features such as:

- ✓ It must contain functional groups that facilitate non-covalent interactions such as hydrogen bonding, electrostatic interactions, π - π stacking, etc.
- ✓ The molecule should be amphiphilic to have an optimum balance between hydrophobic and hydrophilic interactions.
- ✓ These non-covalent interactions must be directional, promoting the formation of anisotropic nanoarchitectures. Hence, most of the LMWGs are chiral molecules.

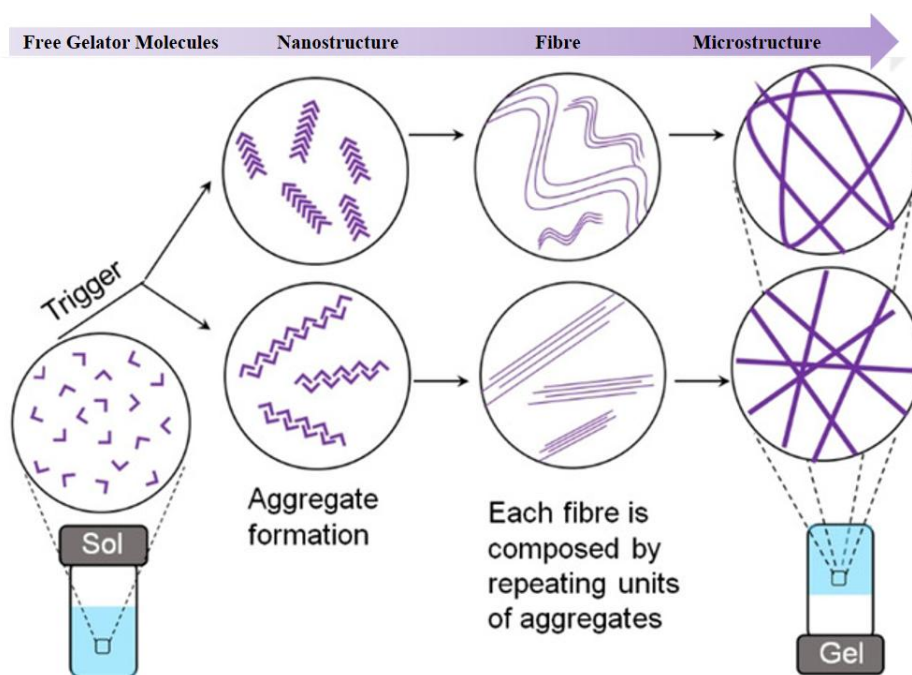


Figure 1.4 Schematic representation of multi-level self-assembly for the formation of supramolecular hydrogel. [Adapted from reference 71]

These molecules self-assemble to form anisotropic one-dimensional supramolecular structures, which form the primary framework of the gel network (**Figure 1.4**). It is presumed to crosslink via physical entanglement, the generation of insoluble aggregates, and chain branching [71]. Depending on the structural properties of LMWG, their self-assembly can be triggered by several external stimuli such as pH, temperature, light, etc [68, 72-74]. These LMWG-based gels are generally stiff and stimuli-responsive. Since the crosslinking is based on dynamic non-covalent interactions, they often show reversible sol-gel transition upon heating and cooling cycles. These dynamic interactions also endow self-healing ability to the hydrogel. It offers several advantages as compared to polymeric gels, including:

- Consistent synthetic process, thereby minimizing the batch-to-batch variation.
- Facile synthetic process involving non-covalent interactions preserves the functional identity of the precursors in the hydrogel material.

Despite significant advances in this field, a key unresolved challenge lies in developing a predictive understanding of whether a molecule will form a gel, precipitate, or crystallize under specific conditions. While many rational approaches and prerequisites are often proposed, gelators are frequently discovered serendipitously [75,76]. In this regard, a diverse array of LMWGs, such as tetrazoles [77,78], pyridines [79,80], carboxamide [81], amino acids [82-84], peptides [85-87], carbohydrates [88], and other small organic molecules, has been extensively explored.

1.5 Hybrid Organic-Inorganic Hydrogels with Intriguing Features and Versatile Applications

Organic-inorganic hybrids represent the unique convergence between two distinct realms of chemistry, combining the complementary advantages of both. It is an innovative strategy to

fabricate new and advanced multifunctional materials with intriguing amalgamated features [27,89]. Beyond retaining the intrinsic features of their individual components, the hybrid material exhibits novel characteristics resulting from the synergistic interactions of both components, such as enhanced electrical and optical properties, chemical and thermal stability, etc. In this regard, a diverse array of such materials, including metal organic framework (MOF), covalent organic framework (COF), metal complexes, and metallogels, has attracted significant attention from the scientific community [90,91]. Their multifunctionality and structural versatility have positioned them at the forefront of innovation across diverse fields of science. Within this extensive landscape, we are confining ourselves to the metallogels, given their central relevance to this thesis.

Metallogels

Metallogels represent an important subclass of gels that contain both metal ions and gelators in their matrix [92]. The incorporation of metal ions in the gel leads to the infusion of properties of metals with the unique features of the gel, thereby widening their scope and utility in versatile applications [35,93,94]. The earliest known metallogel can be traced back to a lithium urate-derived hydrogel reported in the mid-19th century [95]. However, modern research into metallogels began to accelerate in the early 21st century. A notable contribution by Xu and co-workers marked a turning point, as they reported the first Pd²⁺ based metallogel using calix[4]arene bearing pyridyl moieties, facilitating the metal binding [96]. Since then, an exponential rise in research activities has been seen in this field, reflecting its expanding scientific relevance and application potential. The design of metallogels has been driven by a persistent quest for suitable ligands capable of directing gel formation. However, the structural diversity of both the ligands and metal ions presents a significant challenge to pinpoint a universal predictive strategy to design a metallogel [93,97,98]. Based on the type of gelator moieties, the metallogels are broadly classified into two categories: molecular metallogels and coordination polymer gels.

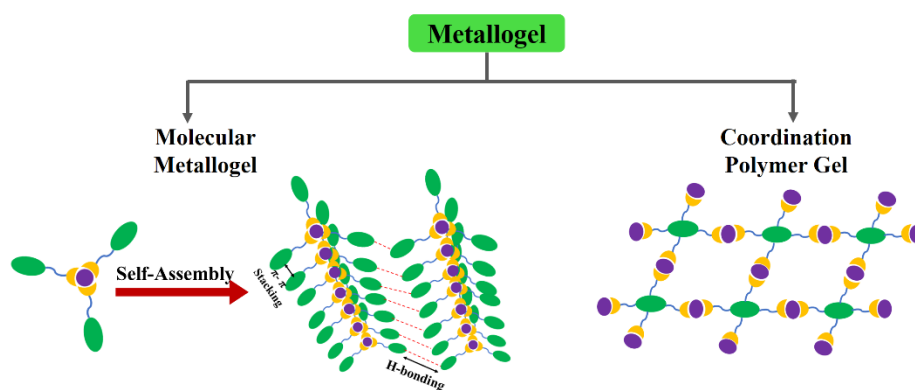


Figure 1.5 Pictorial representation depicting classification of metallogels.

Molecular Metallogels: These are a class of metallogels where discrete metal complexes or units act as gelators; however, metal coordination is inert to the formation of the gel matrix (**Figure 1.5**) [99,100]. Molecular building blocks can be metalated to introduce specific functionalities associated with metal ions and bound strongly within the gelator framework. These metalated gelators often exhibit strong and directional supramolecular aggregation. The gelation process is predominantly governed by the non-covalent interactions, like hydrogen bonding, π - π stacking, van der Waals, and hydrophobic interactions. In this case, metal is an integral part of the gelator, but not directly involved in the gel formation. These gels exhibit stimuli-responsive behaviour and thermoreversible properties similar to the conventional organogelator analogues.

Coordination Polymer Gels (CPG): This class of gels is an emerging category of metallogels, where metal ions act as nodes and gelators as linkers (**Figure 1.5**). In such cases metal ion is an integral part of the gel or the fibrous network, and the metal-ligand interaction is the primary interaction that induces the gelation [19,35]. The initial step to fabricate a coordination polymer gel involves the rational design of the gelator moiety containing at least two metal-binding sites. Metal-gelator binding induces the self-assembly to form coordination polymers, which further assemble to form a 3D entangled fibrous network leveraging the non-covalent interactions. The functional properties of the resultant CPG are highly dependent on the coordination geometry of metal ions,

the structure of gelators, etc. Hence, molecular designing of gelators and selection of metal ions are crucial to modulate the structural and functional properties of CPGs. Coordinated metal ions impart CPGs with unique redox, optical, electronic, and catalytic properties. Their facile fabrication strategy and dynamic nature make them a promising candidate for applications in catalysis, electrocatalysis, biomedicine, and nanoelectronics (**Figure 1.6**).

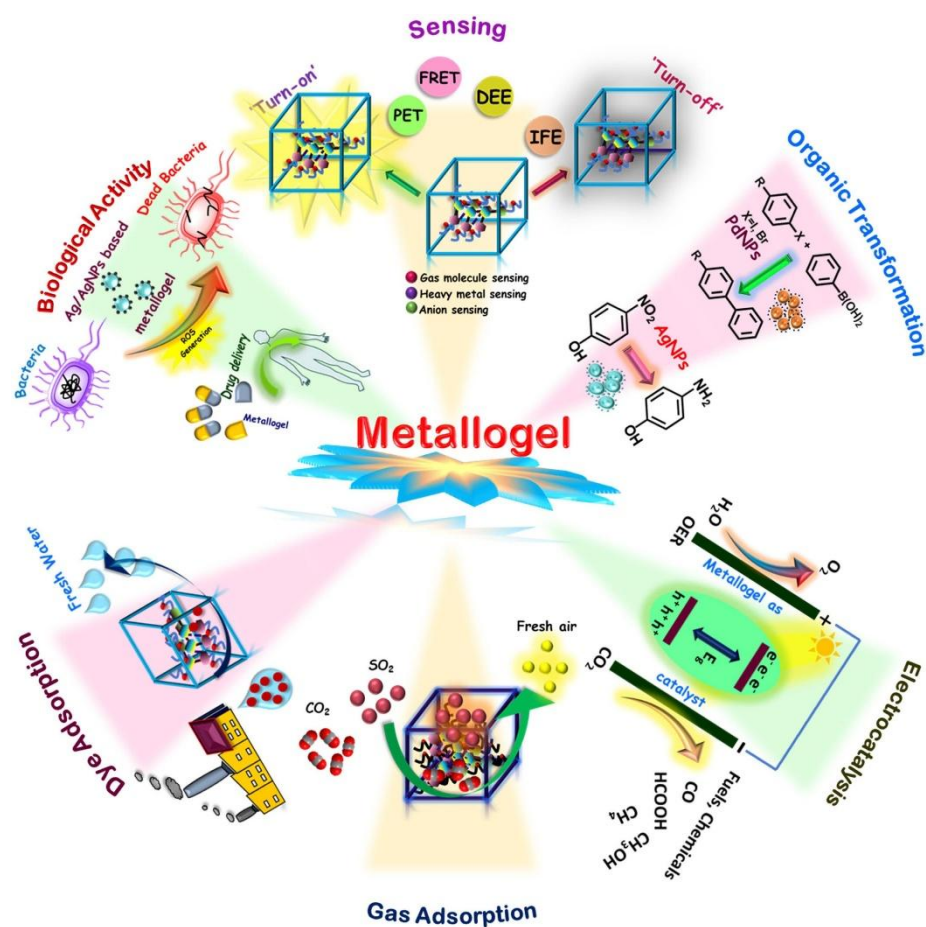


Figure 1.6 Applications of metallogels in diverse fields of science. [Adapted from reference 91]

1.6 From Molecules to Networks: Factors Driving Multilevel Self-assembly of Gels.

There are several factors that influence gel formation. No single parameter can decide the fate of a gel, but it is often the optimum and simultaneous contribution from all the factors that leads to gelation (**Figure 1.7**). While this complicates the understanding, the properties

of the resulting gels are fundamentally dictated by the specific pathway of their assembly [68].

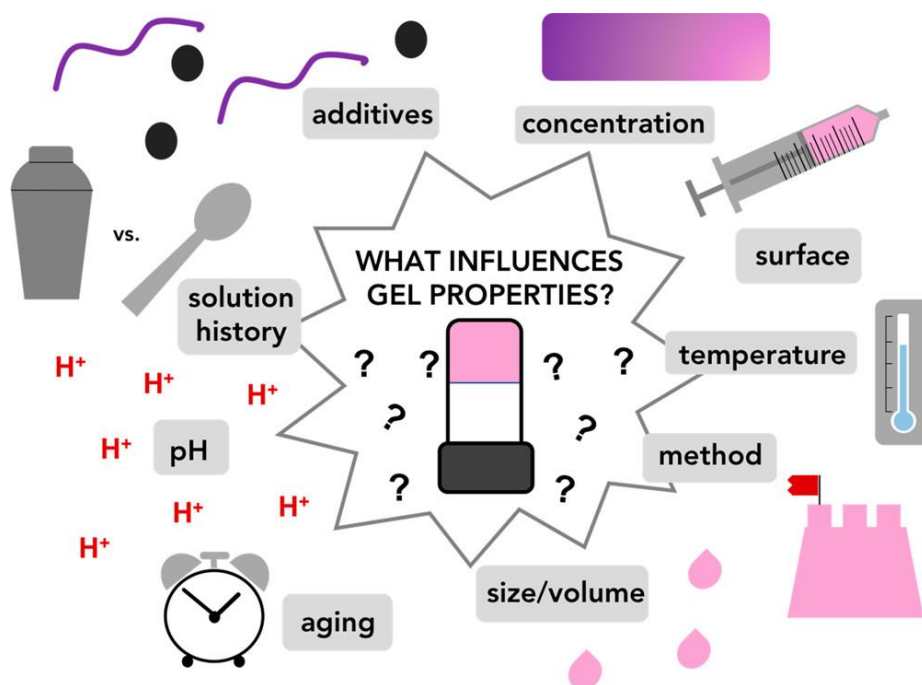


Figure 1.7 Factors influencing gel formation. [Adapted from reference 72]

Hydrogel represents a higher-order self-assembled system. So, we will further highlight the three levels of assembly involved in gel formation and their importance in understanding the self-assembly process (**Figure 1.8**) [23,68,76].

Molecular-Level Assembly: The first level of assembly occurs at the molecular scale, where non-covalent interactions drive the proximity of individual units. Although deciphering these interactions remains challenging, certain characterization techniques can provide a broader overview of the assembly, albeit without resolving the precise nature of the interactions. Several spectroscopic techniques, such as Infrared (IR), Nuclear Magnetic Resonance (NMR), UV-visible, and fluorescence spectroscopy, can help to investigate the hydrogen bonding and other molecular interactions. X-Ray diffraction (XRD) studies provide insights into the assembled structure along with the interplanar distance of π - π stacked layers.

Fibre-Level Assembly: Following molecular-level assembly, gelation typically proceeds via the formation of extended anisotropic architectures, such as nanofibers or nanotubes. This hierarchical organization is commonly probed using microscopic techniques like scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM), providing the visual evidence of the nanoscale morphology.

Network-Level Assembly: The final level of assembly occurs at the network level, where fibres entangle together. The nature of crosslinking critically influences the properties of gels. Although the cartoon representation of gel is always homogeneous, this is not always the case. To avoid drying issues, confocal microscopy is often employed, where imaging can be done in the hydrated state by using a fluorescent probe. Gelation methods directly influence the entanglement, shape, size, and density of the fibrous network [72,101].

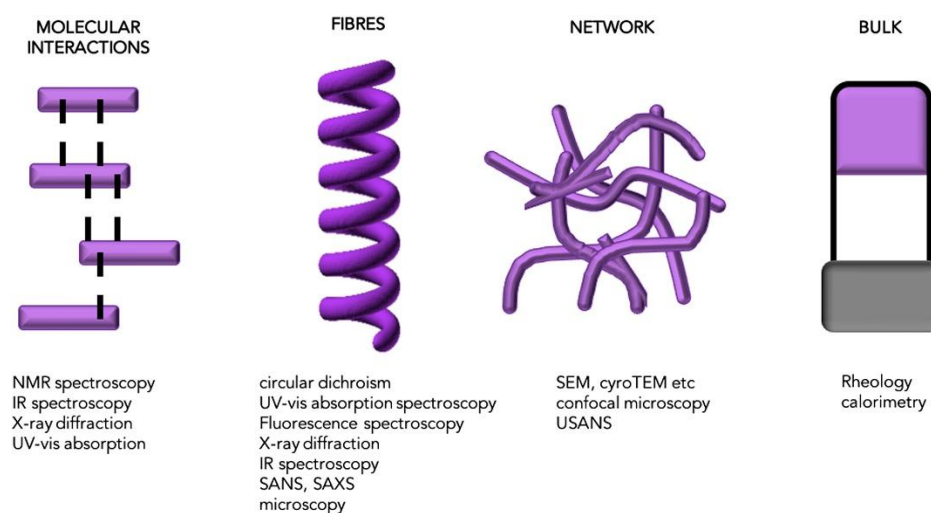


Figure 1.8 Various characterization tools to understand the self-assembly process of gelation. [Adapted from reference 68]

Finally, the mechanical properties of gels are evaluated using rheology, which provides quantitative insights into the underlying network structure. Several rheological measurements can elucidate key features such as the coexistence of two phases and the viscoelastic behaviour of gels. The storage modulus (G') is dominant over the loss

modulus (G''). Typical LMWG-based gels exhibit frequency-independent storage and loss moduli.

1.7 Small Biomolecules as Low-Molecular-Weight Gelators: A Bioinspired Approach to Functional Soft Materials

1.7.1 Nucleic acid derivatives as LMWG

In contrast with the different LMWGs discussed above, several supramolecular hydrogels have been fabricated by leveraging the unique self-assembly and molecular recognition properties of the nucleic acid derivatives. Among other nucleobases are nitrogen-containing heterocycles serving as the basic units of nucleosides, nucleotides, and nucleic acids. Nucleobase-containing molecules hold fundamental biological significance, with nucleic acids and G-quadruplex DNA/RNA structures playing critical roles in key cellular processes [102,103]. Among the two categories, purine and pyrimidine, the bicyclic structure of purines provides multiple facets for hydrogen bonding, while their aromatic character facilitates efficient π - π stacking interactions (**Figure 1.9**).

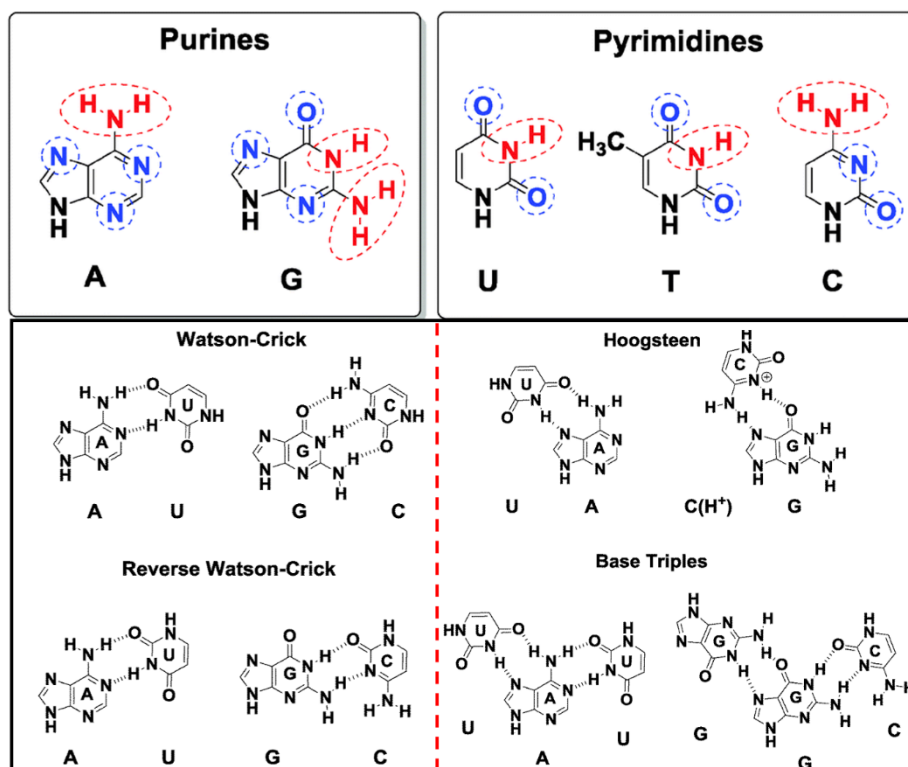


Figure 1.9 Chemical structure of different nucleobases and the different modes of base pairing. [Adapted from reference 103]

Among the other non-covalent interactions exhibited by nucleobases, the most canonical is Watson-Crick base pairing, serving as the fundamental recognition motif in double-stranded nucleic acids. While this hydrogen bonding arrangement is most studied and well known, it represents only a subset of all the possible interactions. Beyond the canonical base pairing, nucleobases are capable of forming homodimers [101,104,105]. Purine nucleobases exhibit a pronounced propensity for self-assembly, attributed to their favourable structural design, which facilitates several non-covalent interactions. These interactions often drive the formation of extended higher-order supramolecular architectures, such as fibres, ribbons, etc., while nucleobases have long served as structural motifs in the design of metal-organic frameworks and functionalization of polymeric backbones, but they have remained relatively unexplored in the context of the hydrogel until the past decade. In this context, several efforts have been made by derivatizing the nucleobases, while our group demonstrated the potential of gel formation by utilizing the native form of nucleobases in the

presence of metals. In 2016, Bhagwati et al. reported the formation of a silver-mediated coordination polymer gel in the presence of all the nucleobases [106]. Their study highlighted the critical role of base pairing, where the mixture of nucleobases resulted in gelation, with the exception of guanine, which could not induce gel formation under similar conditions. Further, Zn^{2+} was utilized to produce metallogels with guanine and cytosine and exploited for photocatalytic degradation of dyes [107]. The formation of in situ CdS quantum dots in the metallogel matrix is formed by the coordination of Cd^{2+} and native nucleobases (Thymine and Uracil) [108]. These works highlight the potential of nucleobases for the fabrication of soft nanomaterials for versatile applications.

Nucleosides are formed through the covalent attachment of a nucleobase to a ribose unit via an N-glycosidic bond, while phosphorylation of these nucleosides results in the formation of nucleotides. The ribose sugar in these biomolecules plays a crucial role in facilitating supramolecular organization.

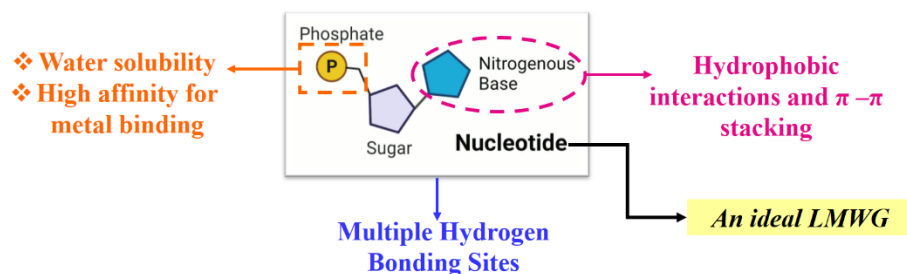


Figure 1.10 Pictorial representation of nucleotide highlighting all the structural features required for hydrogelation.

Gelation by these molecules often requires hydrophobic modification of the nucleobase or the sugar moiety to introduce the optimum hydrophobicity [109,110]. Some simple derivatized nucleosides have been explored as the LMWG, such as diacylated uridinophosphocholine derivative, benzyltriazole appended deoxyuridine, etc [111,112]. These works clearly highlight the balance between the hydrophobic and hydrophilic character of the gelator in order to induce hydrogelation. In this context, Oda *et al.* reported the

gelation ability of gemini surfactant upon mixing with equimolar solutions of adenosine and 5'-uridine monophosphate (5'-UMP), where the complementary base pairing between A-U governs the self-assembly [113]. Among all other nucleosides, guanosine is uniquely distinguished in terms of its ability to form higher-order G-assemblies or superstructures owing to its special structural features. This aspect will be discussed in detail later on. So far, we have understood how nucleobases and nucleosides fulfil all the structural requirements for being ideal low-molecular-weight gelators.

In line with the scope of the thesis, we extend our discussion to the exploration of nucleotides as the gelator, particularly to purine nucleotides. The presence of an anionic phosphate group provides additional electrostatic interactions and acts as a hydrogen bond acceptor. Moreover, the high affinity of the phosphate group for metal ions significantly broadens the utilization of nucleotides as LMWG within the realm of metallogels (**Figure 1.10**). In this regard, several researchers are trying to develop novel hydrogel systems using different nucleotides and their derivatives [114-121].

1.7.2 The Self-assembled Aggregates of Guanosine Derivatives

We have already mentioned that guanine has unique structural features among other nucleobases. Guanosine is one of the important building blocks, among other natural nucleosides, to develop higher-order self-assembled architectures. Guanosine has more number of self-complementary hydrogen bonding sites: three hydrogen bond acceptors (N3, N7, and O6), and two hydrogen bond donor groups (N1 and N2), which helps to form superstructures like quartets, dimers, etc., indicating its supremacy [122-125]. Apart from the well-known Watson-Crick base pairing, it can also form Hoogsteen H-bonding. The four G units non-covalently interact using eight intermolecular H-bonds. This special structural arrangement is popularly known as G-quartets (G4), formed under the appropriate pH and concentration. The presence of specific cations such as K^+ , Na^+ , Li^+ , Ca^{2+} , etc, promotes the formation of G-

quartets by minimizing the electrostatic repulsion between the carbonyl groups of guanine base that point inward within the inner cavity of the quartet (**Figure 1.11**) [126,127].

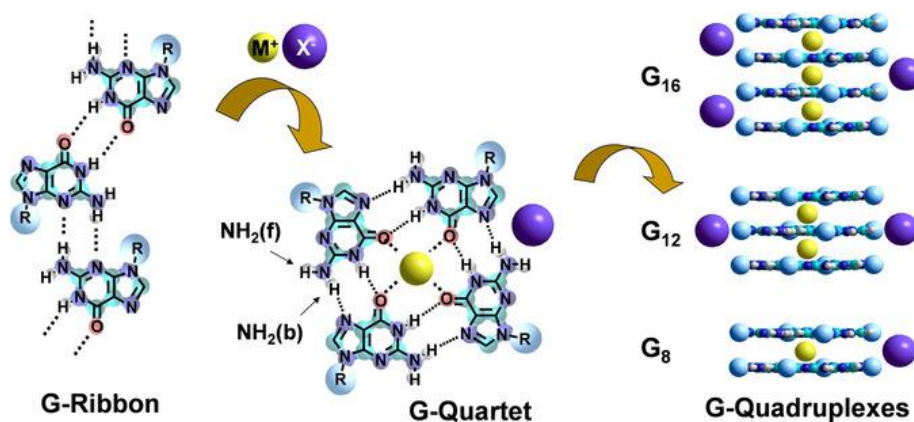


Figure 1.11 Representation of different G-assemblies [Adapted from 105]

The optimum contribution of eight hydrogen bonds, delocalization of nucleobase electrons, and cation-mediated electronic stabilization led to the stability of this macrocyclic structure. These individual G-quartet stacks on top of each other to form a columnar type of assembly, called as G-quadruplex and G-wires [105]. G-quadruplexes are mostly found in DNA and some guanine-rich RNA. Being an inherent part of the genetic material, G-quadruplex serves crucial roles in several biological functions, including gene regulation, DNA replication, transcription, etc. Owing to its inherent biocompatibility and biodegradability, the self-assemblies of guanosine and its derivatives are emerging as promising candidates in diverse fields of science, such as material sciences, nanotechnology, biomedical, and supramolecular chemistry [128-130].

1.7.3 Peptides as key crosslinkers and gelators

Apart from nucleobases and nucleotides, short peptide-based supramolecular hydrogels are often the ideal candidate considered for the fabrication of soft materials. Among them, short peptides are preferred for constructing hierarchical nanoarchitectures due to their synthetic accessibility, biocompatibility, and a simple model to

investigate the mechanistic insights underlying at the core of the complex self-assembly in nature [131-133].

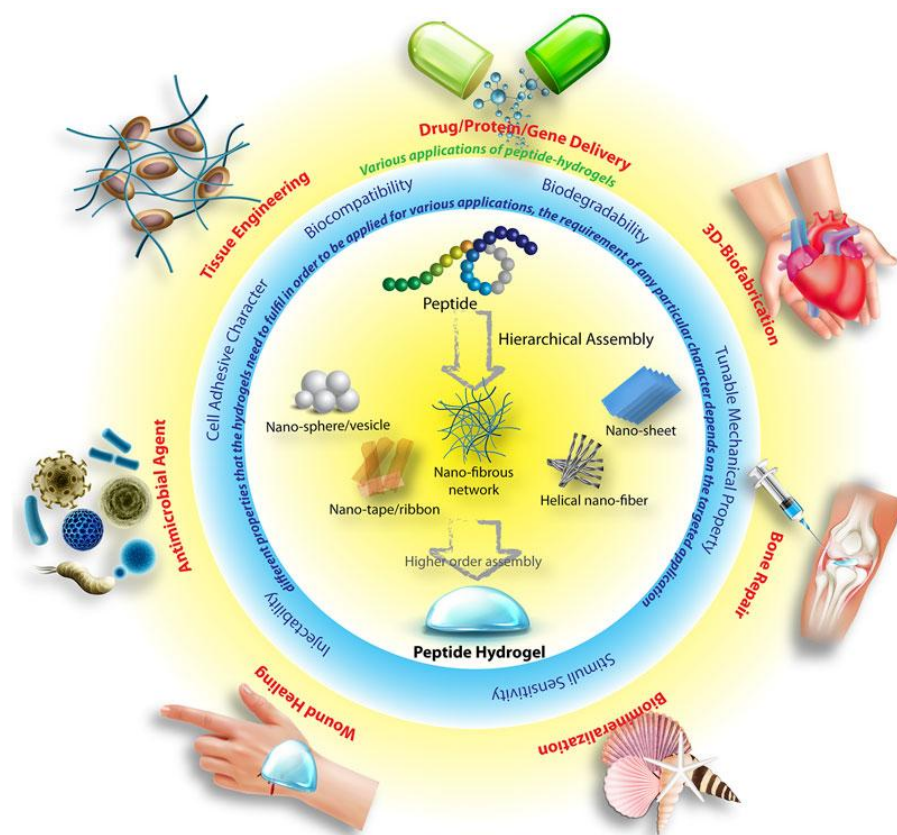


Figure 1.12 Multidimensional applications of peptide-based hydrogel. [Adapted from reference 134]

The precise balance between hydrophilic and hydrophobic interactions is fundamental in guiding peptide self-assembly into hydrogels. In this regard, the aromatic amino acids such as tyrosine, phenylalanine, and tryptophan are often used to design the peptide-based gelators to mediate π - π stacking interactions. However, additional aromatic protecting groups can also be anchored to promote hydrophobicity and other non-covalent interactions. Several peptide-based gelators such as Fmoc-peptides, Lysine, pyrene, pyridine, and naphthalene modified peptides, oligopeptides, nucleopeptides, etc, have been explored as LMWGs to fabricate soft smart materials. The inherent antimicrobial, antiviral, anti-inflammatory, and antioxidant properties of numerous peptides position them as ideal scaffolds for a wide range of biomedical applications (**Figure 1.12**) [134-137].

1.8 Recent Advancements in Purine Nucleotide-Derived Functional Hydrogels

Although there is a growing interest in nucleotide-based hydrogels owing to their multifunctionality and other unique features, the field remains relatively nascent. These systems offer immense potential for the development of biocompatible and biomimetic smart materials. Moreover, their ability to interact with metal ions and other inorganic components makes them suitable for a broad spectrum of advanced and versatile applications. This thesis work is confined to the exploration of AMP and GMP (purine nucleotides) as LMWGs for the fabrication of multifunctional soft materials. In this section, we will discuss the recent developments in the design, assembly, and functional applications of nucleotide-based hydrogels. Guanosine and its derivatives are well-known gelators due to their ability to form higher-order G-assemblies, whereas the self-assembly and gelation behaviour of adenosine monophosphate is relatively unexplored. Although the formation of coordination polymer particles using AMP has been documented, reports elucidating its gelation propensity are very limited and are discussed in the following section.

1.8.1 Advances in AMP-assembled functional hydrogels for versatile applications

Adenosine is present in several important bioactive biomolecules, including ATP and cyclic adenosine monophosphate (cAMP). Among other nucleotides, recognition of AMP is vital as it serves crucial roles in metabolism, transfer of genetic information, and bioenergetics. In 2012, Du *et al.* first reported the formation of a hydrogel using catalytic dephosphorylation of AMP, where an adenosine and AMP-based precursor was synthesized with a tripeptide derivative Phe-Phe-Lys (NapFFK) [138]. Upon addition of alkaline phosphatase to the precursor solution, enzymatic dephosphorylation generated a

hydrogelator that spontaneously self-assembled into a hydrogel (**Figure 1.13a**). This study highlighted the significance of AMP as a functional molecule and a potential template for the development of novel biomaterials.

Further, in 2015, Gupta and coworkers fabricated the AMP-templated β -FeOOH hydrogel. The AMP-modified colloidal β -FeOOH nanostructures were synthesized by adding FeCl_3 to the aqueous solution of AMP. Upon refluxing the solution at 100 °C for 6 h, the formation of colloidal samples was observed. At higher concentrations, the colloidal solution transformed into a hydrogel within a period of 10 days. The magnetic studies revealed that the coating of AMP enhanced the magnetization and superparamagnetic behaviour of these nanostructures at room temperature (**Figure 1.13b**) [139]. This study marked a profound impact, positioning AMP as a multifunctional molecular template. After this few metallogels were developed by exploiting AMP as a low molecular weight gelator. Liang *et al.* reported the first Zn^{2+} based metallogel, where the addition of Zn^{2+} to the AMP in a specific ratio resulted in gel formation [140]. It was highly specific, as other nucleotides like GMP and CMP failed to induce gelation (**Figure 1.13c**). The hydrogel showed self-healing behaviour and nanofibrillar morphology. The Zn^{2+} -AMP hydrogel showed the encapsulation of guest molecules and can be utilized in wastewater management. This paved the way for the advancement of metallogel for versatile applications.

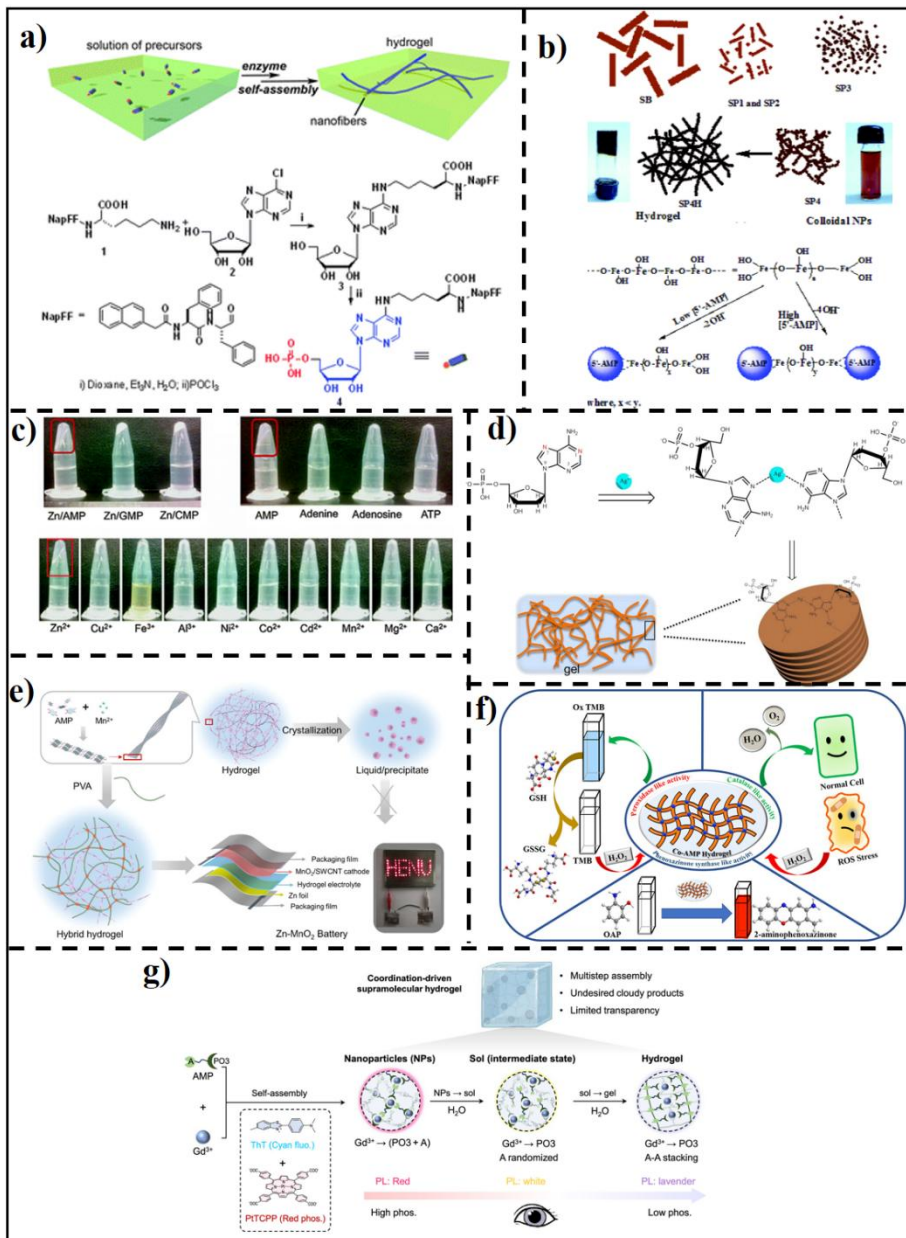


Figure 1.13 a) ALP catalyzed hydrogel formation using adenosine, AMP, and tripeptide-based hydrogelator [138], b) β -FeOOH modified AMP as a template for hydrogel formation, showing enhanced magnetic behaviour[139] (copyright RSC 2015), c) Zn^{2+} -AMP metallogel for the guest molecules encapsulation [140] (copyright RSC 2015), d) Ag^+ mediated self-assembly of AMP leading to hydrogel formation and in situ Ag nanoparticle formation upon exposure to UV and visible light [141] (copyright RSC 2017), e) Mn^{2+} -AMP/PVA hybrid hydrogel for the energy storage devices [142] (copyright ACS 2020), f) Co-AMP heat-set hydrogel for pH-dependent multinanozymatic activities and mitigation of cellular oxidative stress [143] (copyright ACS 2023, g) Gd^{3+} -AMP hydrogel exhibiting RTP for the visual detection of the self-assembly dynamics [144] (copyright ACS 2024).

In this context, Hu *et al.* developed a pH and light-sensitive Ag⁺-AMP supramolecular hydrogel [141]. Interestingly, in situ Ag nanoparticles were produced when the hydrogel was exposed to visible light or UV light due to photoreduction (**Figure 1.13d**). The nitrogen-rich moieties in AMP facilitate the photoreduction process. The gel can be compacted by centrifugation while it readily reforms by vortexing. This property was exploited for the efficient encapsulation and enrichment of important guest biomolecules. The formation of nanoparticles inside the gel matrix also widens its utility in the field of catalysis. In 2020, another Mn²⁺-AMP hydrogel was developed by Zhai and coworkers with a nanofibrillar morphology [142]. Polyvinyl alcohol (PVA) was added to enhance the mechanical strength of the hydrogel. AMP-Mn²⁺/PVA hydrogel was used as the hydrogel electrolyte in Zn-MnO₂ batteries, and showed excellent electrochemical stability (**Figure 1.13e**). In addition to this, Sarma and coworkers reported the formation of a Co-AMP heat-set metallogel [143]. Interestingly, the hydrogel showed remarkable pH and thermal stability. The facile synthetic protocol coupled with its exceptional stability rendered the hydrogel particularly attractive. The hydrogel showed pH-dependent multienzymatic activities (**Figure 1.13f**). The catalase mimicking activity was utilized to mitigate the oxidative stress generated in cells upon exposure to H₂O₂. Very recently, Wang *et al.* developed a Gd³⁺-AMP hydrogel that showed room temperature phosphorescence (RTP)-inducing properties [144]. They showed the AMP concentration-dependent NPs to gel transition, and the inclusion of appropriate dye molecules coupled with RTP enabled direct visualization of the dynamics of self-assembly through the naked eye (**Figure 1.13g**). This work provided critical insights into coordination-driven self-assembly in gelation, assisting the synthesis of supramolecular hydrogel in a controlled manner. These notable works in the field highlighted the importance of developing AMP as an LMWG for the fabrication of soft and smart materials. This thesis work elucidates the potential of utilizing a single hydrogel framework as a multifunctional platform for

applications ranging from biomimetics to electrocatalysis in the subsequent chapters.

1.8.2 Recent progress in GMP-inspired hydrogels as a multifunctional platform for broad-spectrum applications

Guanosine and derivatives hold special interest owing to the formation of higher-order G-assemblies. This field has started a long way back since the 19th century, when a German scientist, Ivar Christian Bang, reported the hydrogel formation from guanylic acid at lower pH. This was the first G-assembly-based hydrogel. About 50 years later, Martin Gellert *et al.* established the structure of G-tetrad by analyzing the XRD pattern of the 5'-GMP gel. Initially, guanosine tetramer formation was regarded as an *in vitro* phenomenon, thereby limiting the research activities on DNA G-quadruplexes for several years. However, by the late 1980s, several evidences revealed the crucial roles of G4 motifs in the genetic process. This recognition spurred extensive biophysical investigations into G4-rich DNA, proving the existence of G-tetramer structures in biological systems. Inspired by nature, scientists have sought to harness these self-assembled systems for the development of smart materials. Though this field has garnered significant attention, it is still a relatively new field. Hydrogels derived from guanosine and its analogues constitute a foundational class within the landscape of G-quadruplex-based materials. However, the employment of GMP to engineer biocompatible smart materials holds immense potential with a broad range of applications. In this regard, some representative recent studies are outlined below to highlight the importance of GMP-based hydrogels for diverse applications.

G-quadruplex hydrogels are highly sought-after materials in the biomedical and biomimetic field owing to their inherent biocompatibility and biodegradability. In 2021, Patil and coworkers developed a stimulus-responsive nucleotide-amino acid hybrid hydrogel generated by Ag⁺-triggered formation of Ag-GMP dimers to produce nanofilaments [121]. Addition of an amino acid (N-

fluorenylmethyloxycarbonyl tyrosine) to the assembly resulted in the formation of a hydrogel. Two distinct kinds of supramolecular nanofilaments were observed upon thorough characterization. This strategy opened up new avenues to engineer novel hybrid supramolecular hydrogels using GMP as a template for biomedical and biological applications. In 2019, Sarma and coworkers reported the formation of a Fe-GMP hydrogel, which was further crosslinked with Ca^{2+} ions to achieve enhanced mechanical strength and biocompatibility (**Figure 1.14a**) [145]. The dual crosslinked Ca-Fe-GMP hydrogel was stimuli-responsive, injectable, and showed a pH-responsive zero-order Dox release profile. The sustained, controlled, and pH-responsive drug release was achieved, underscoring its importance for several biological applications. In 2022, an all-biomolecule hydrogel was developed by Cheng *et al.*, where they fabricated a GMP-based hydrogel by utilizing the antibacterial drug tobramycin (TOB) [146]. The hydrogel was used for treating keratitis. The all-biomolecule system was biocompatible, injectable, transparent, and showed sustained drug release. Interestingly, the treatment with pristine drug led to the formation of an opaque layer on the cornea, while it was transparent upon treatment with hydrogel (**Figure 1.14b**). Moreover, the therapeutic efficacy of the drug was also enhanced. This work highlighted the synergistic role of GMP as a template for efficient drug carriers.

Further, in 2023, a multicomponent hydrogel was developed by using guanosine (Guo) and GMP for wound healing, leveraging 3D bioprinting and tissue reconstruction [147]. They utilized the strong crosslinking of Guo and GMP with the boronic acid and K^+ ions to enhance the mechanical strength and stability of the hydrogel (**Figure 1.14c**). In 2024, our group reported the biocondensation of biomass and DNA, which self-assembled to form a hydrogel at higher concentrations [148]. This all-nucleic hydrogel was used as a hydrotropic drug carrier with high encapsulation efficiency of both the hydrophilic and hydrophobic drugs (**Figure 1.14d**). It offered a new strategy of utilizing biomass DNA for the fabrication of soft materials.

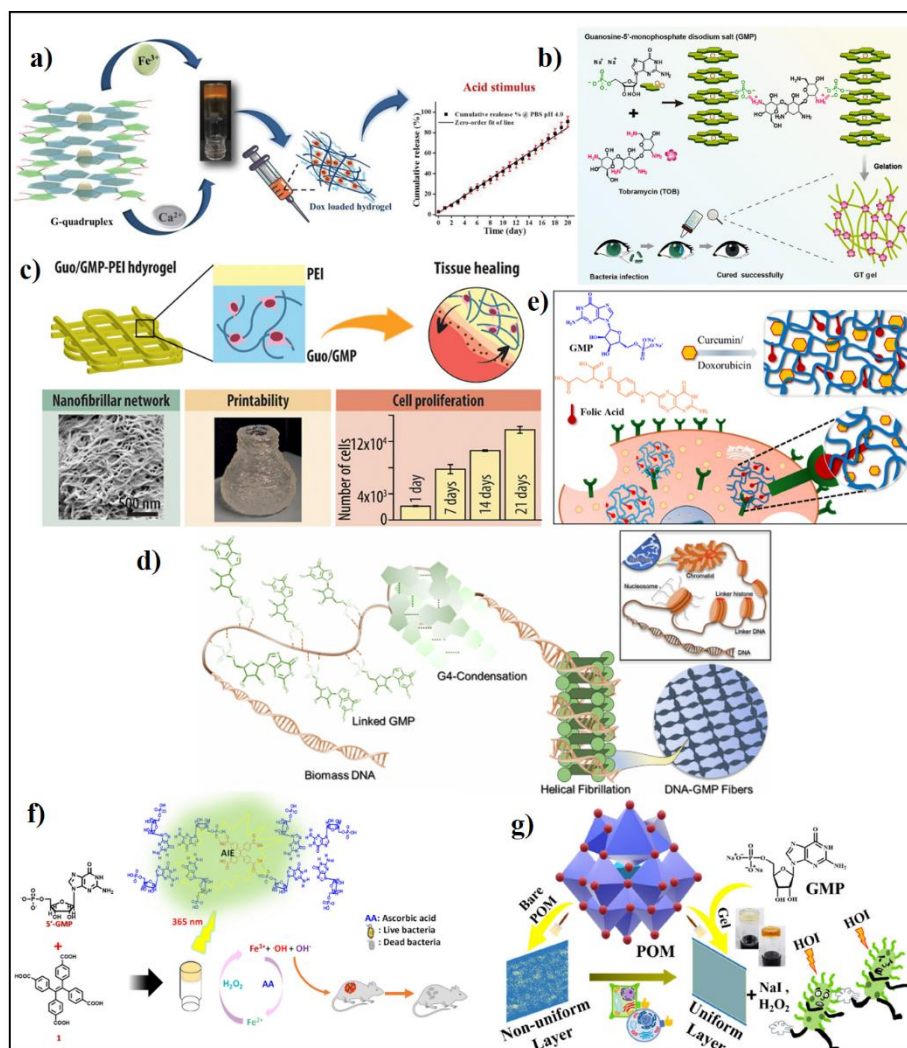


Figure 1.14 a) Ca-Fe-GMP hydrogel for pH-responsive drug delivery [Adapted from 145], b) GMP-TOB hydrogel for the treatment of keratitis [Adapted from 146], c) Guo/GMP hydrogel for tissue healing using bioprinting [Adapted from 147], d) DNA-GMP hydrogel as a hydrotropic carrier for both hydrophilic and hydrophobic drugs [Adapted from 148], e) Folic acid-GMP hydrogel for target drug delivery of anticancer drugs [Adapted from 149], f) GMP-tetra(4-carboxyphenyl) ethylene metal incorporated hydrogels for the treatment of dorsal skin infection [Adapted from 150], g) POM-GMP hydrogel showed haloperoxidase mimicking activity and antifouling activity [Adapted from 151].

Recently, our group also demonstrated that target drug delivery can be achieved through the strategic selection of crosslinking precursors with GMP. In this work, folic acid and GMP were crosslinked to form a supramolecular hydrogel and exploited for the sustained drug delivery of hydrophilic (Dox) and hydrophobic (Curcumin) drugs [149]. The hydrogel was self-adhesive, biocompatible, and the target drug

delivery was achieved by leveraging the overexpressed folate receptors in the cancer cells. This strategy could enhance the efficiency of the drug while minimizing its adverse side effects (**Figure 1.14e**). Another GMP hydrogel was reported by Pal and coworkers, where GMP and tetra(4-carboxyphenyl) ethylene were used to fabricate the hydrogel [150]. The addition of divalent ions like Fe^{2+} , Mg^{2+} , and Ca^{2+} tuned the mechanical strength and aggregation-induced emission behaviour of the hydrogel. Furthermore, the Fe-GMP hydrogel exhibited antibacterial properties owing to its in situ ROS generation capacity. In vivo studies suggested the antibacterial efficacy of hydrogel by treating the dorsal skin infection in mice (**Figure 1.14f**). This highlights the applicability of these hydrogels for several biological and biomedical applications.

The field of enzyme mimetics is gaining attention, and in this regard, Chakraborty *et al.* reported polyoxometalate (POM)-GMP hydrogels with haloperoxidase mimicking activity. The employment of GMP rendered a multifold enhancement in the biocompatibility of the hydrogel [151]. The antifouling activity of the POM-GMP hydrogel was evident with both the Gram-negative and Gram-positive bacteria. The activity of the hydrogel was increased as compared to its bare POM counterparts (**Figure 1.14g**). This work highlighted the design and development of robust antifouling coatings with minimal synthetic requirements. Another POM-GMP-based hydrogel was designed by the same group, where phosphomolybdic acid was self-assembled with GMP using a facile synthetic strategy [152]. The hydrogel showed multichromic properties against several stimuli such as light, stress, heat, electricity, and metals. The bare POM did not exhibit multichromism, which was manifested only upon the formation of a supramolecular network. A 2-layered electrochromic device was designed owing to the high electrical conductivity of the gel matrix. This strategy offered a simple and low-cost alternative to a conventional 5-layered setup. These works demonstrated how inorganic crosslinkers can be leveraged to design multifunctional GMP-based hydrogels, while infusing a variety of applications.

1.9 Motivation and Organization of the Thesis

This thesis aims to establish a green, facile, and straightforward synthetic strategy for the engineering of supramolecular soft hybrid materials. The proposed methodology is devoid of any chemical modification, thereby preserving the native state of the biomolecule in the biomaterials. By circumventing the covalent modifications, this strategy not only eliminates tedious lab procedures but also ensures the intrinsic properties of the precursor molecules remain intact. The purine nucleotides, AMP and GMP, were employed as LMWGs to fabricate self-assembled hydrogels through several non-covalent interactions. These systems highlight the versatility of pristine nucleotides to form hybrid materials through their crosslinking with inorganic as well as organic moieties. It enables the design and development of numerous hydrogels with desirable functional properties through the strategic selection of precursors. This thesis also showcases how a single gel matrix can be used as a multifunctional platform, underscoring the potential of hydrogels as a pivotal class of emerging soft materials with broad applicability across diverse scientific domains. These nucleotide-hydrogels can serve as a biocompatible template for several biological and biomedical applications. The thesis is broadly divided into two parts, where the first 2 working chapters comprise synthesis, characterization, and multidimensional applications of the cobalt-adenosine monophosphate hydrogel, whereas the second part focuses on the design and development of glutathione-GMP hydrogels for biomedical applications.

Chapter 2 discusses the synthesis of a cobalt-based coordination polymer hydrogel using adenosine monophosphate as the LMWG. The gelation is accompanied by a color change from pink to blue, indicating a structural transition from octahedral to tetrahedral around the cobalt centre. The hydrogel is a heat-set gel and is stable in a wide pH window from 1 to 12. The hydrogel has a longer shelf life and can be stored under ambient conditions. The hydrogel was mechanically robust and showed

thixotropic behaviour. The Co-AMP hydrogel was characterized using several spectroscopic and microscopic techniques. Magnetic studies suggest the superparamagnetic behaviour of hydrogel at low temperature.

Chapter 3 presents the versatile applications of Co-AMP hydrogel in diverse fields of science, ranging from biomimetics to electrocatalysis. This section of the thesis demonstrates the multifunctionality of the single hydrogel matrix. This chapter is divided into three parts, where each chapter discusses the specific application of the Co-AMP hydrogel.

Chapter 3A discusses the exploitation of the Co-AMP multienzyme mimicking system. It showed pH-dependent peroxidase and catalase mimicking activities and functions as a biomimetic analogue of phenoxazinone synthase enzyme. The catalase activity of the Co-AMP hydrogel was used to mitigate the cellular oxidative stress induced by elevated concentrations of H_2O_2 . The hydrogelation with AMP reduces the cytotoxicity of cobalt metal. In vitro cell studies also indicate the potent antioxidant activity of the hydrogel.

Chapter 3B explores the potential of the Co-AMP hydrogel as a carbonic anhydrase (CA) mimicking system. The tetrahedral coordination geometry of Co^{2+} , the presence of the imidazole moiety in AMP, and the nitrogen-rich matrix of hydrogel prompted us to explore the CA mimicking activity of this hydrogel. Notably, this represents the first report of a non-zinc and non-histidine CA mimic as per our knowledge. It exhibits high esterase and CO_2 hydration activity. The CA mimicking activity of the hydrogel facilitated the biomineralization of metals.

Chapter 3C explores the electrocatalytic activity of the Co-AMP coordination polymer hydrogel for overall water splitting in 1 M KOH and industrially relevant conditions. This represents an integrated platform where the synergistic interplay of multiple parameters led to the enhancement in overall catalytic efficacy. The tetrahedral coordination mode of Co^{2+} , the presence of the phosphate group of the

ligand, and the swelling and thixotropic behaviour collectively rendered it as an efficient bifunctional electrocatalyst. Notably, the ease of large-scale synthesis, cost-effectiveness, and exceptional stability of the material make it suitable for industrial implications.

Chapter 4 outlines the synthesis of two glutathione-based hydrogels using GMP as the LMWG. The hydrogels formed spontaneously by the simple mixing of the aqueous solutions of the precursors. These hydrogels are mechanically robust, injectable, stimuli-responsive, and self-healing. Moreover, the native form of biomolecules is preserved in the biomaterials. Considering the biological relevance of glutathione, these hydrogels were used for biological applications. In vitro studies suggest that both hydrogels are biocompatible and possess intrinsic multimodal bioactivities. The hydrogels showed antibacterial activity against both Gram-positive and Gram-negative bacteria, antiviral activity against EBV and SARS-CoV-2. It also showed anticancer activities. The study highlights the multiutility of the nucleotide-glutathione platform in biological and biomedical applications.

Chapter 5 summarizes the relevance of the works included in the thesis and highlights the interdisciplinary potential of nucleotide-based hydrogels. This chapter also describes the emerging role of hydrogels as a versatile class of soft material, while also outlining the future directions and opportunities in the field.

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Chapter 2

Synthesis and Characterization of Supramolecular Self-assembled Cobalt Adenosine Monophosphate (Co-AMP) Hydrogel

2.1 Motivation

The incorporation of metal ions to trigger gelation leads to the blending of the intrinsic properties of metals with the distinctive features of gels, thereby expanding their utility in diverse domains of science. In this chapter, we will discuss the synthetic strategy of a cobalt-based metallogel where AMP is used as the gelator moiety. The limited gelation capability of AMP with specific metal ions is already being discussed in Chapter 1. Here, Co^{2+} ions are utilized as the crosslinker to induce the gelation with AMP. Cobalt is one of the most widely used and researched elements in almost all fields of catalysis. In the last few decades, cobalt-based catalysts have gained considerable interest in the energy and environmental sectors to such an extent that the European Union added it to the list of critical raw materials [1-3].

Cobalt is an essential trace element and is found in several enzymes, including cobalamine (Vitamin B12), methionine aminopeptidase, nitrile hydratase, bromoperoxidase, etc. The cobalt complexes drive many important biological processes, like nitrogen fixation in plants. Therefore, cobalt metallogels with pristine biomolecules hold significant importance for the advancement of hydrogel systems in biological and biomedical applications [4]. The hydrogel is an emerging multifunctional platform for versatile applications. In this context, we developed a cobalt-adenosine monophosphate hydrogel using a facile and straightforward synthetic strategy that is devoid of any chemical modification. The developed

hydrogel showed exceptional pH and thermal stability and can be stored in ambient conditions for years. The Co-AMP hydrogel is being exploited for versatile applications in the field of enzyme mimics, CO₂ fixation, and energy generation applications, which will be discussed in subsequent chapters in detail. This chapter particularly discusses the synthesis and characterization of the Co-AMP hydrogel. Several spectroscopic and microscopic techniques were employed to propose a plausible structure for the Co-AMP hydrogel.

2.2 Synthesis of Hydrogel

0.1M stock solution of CoCl₂·6H₂O and 5'-AMP was prepared by dissolving them in Milli-Q water. Then, both the stock solutions were mixed in a separate vial, maintaining the molar ratio of 1:1 (50 mM). At room temperature, the mixture was pink in color. When the mixture was heated at 60 °C, it initially became turbid and gradually transformed into a blue-colored hydrogel. The gelation was confirmed visually by the vial inversion test, where the solution did not flow under the influence of gravity, indicating the gelation (**Figure 2.1**).

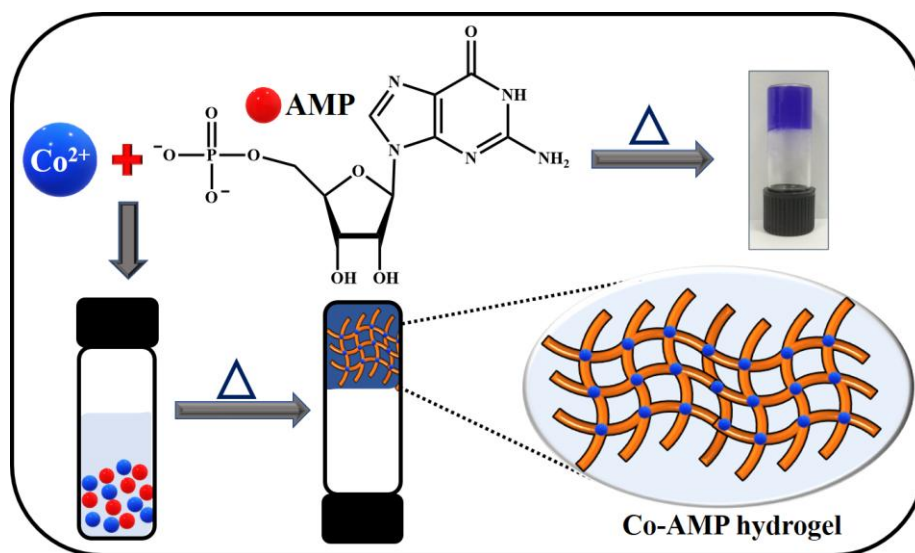


Figure 2.1 Synthetic scheme for the fabrication of Co-AMP hydrogel.

2.3 Properties of Co-AMP Hydrogel

2.3.1 Concentration Optimization of the Hydrogel

The concentration-dependent study was performed with respect to both precursors to obtain the minimum gelation concentration. The concentration of metal (Co^{2+}) was varied while keeping the concentration of AMP constant. It was observed that no gelation occurred when the Co^{2+} concentration was lower than the concentration of AMP in the mixture. A minimum Co^{2+} concentration of 50 mM was required, below which only a viscous solution was obtained. In a similar experiment, the concentration of AMP was varied while keeping the Co^{2+} concentration constant at 50 mM. It was observed that no stable gels were obtained at a lower (less than 30 mM) or higher (above 100 mM) concentration of AMP. Stable gels were obtained at a higher Co^{2+} :AMP ratio (**Figure 2.2a, 2.2b**).

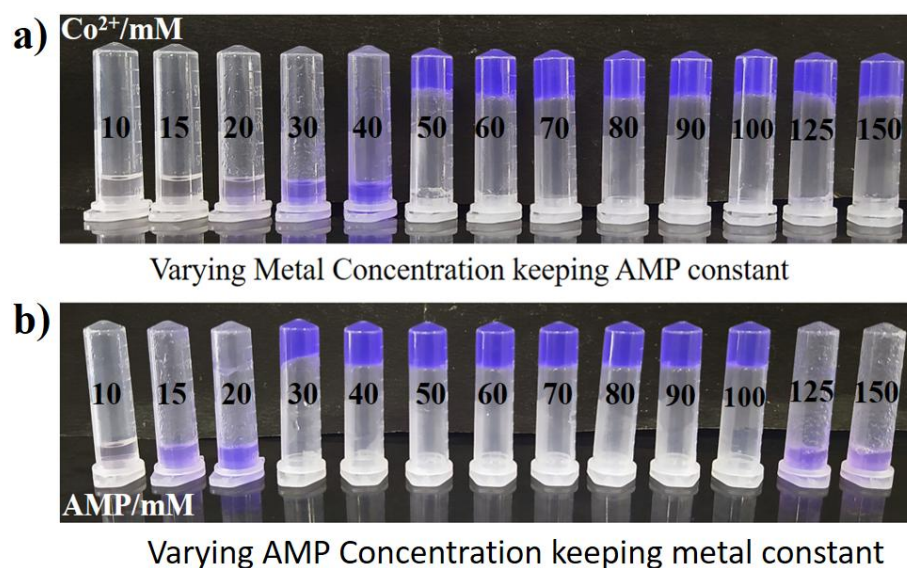


Figure 2.2 a,b) Digital image showing the minimum gelation concentration and effect of variation of concentration of both metal salt and nucleotide.

A Job's plot was obtained by varying the mole fractions of Co^{2+} ions and AMP, which suggested the stable Co-AMP complex formation at a 1:1 molar ratio (**Figure 2.3a, 2.3b**).

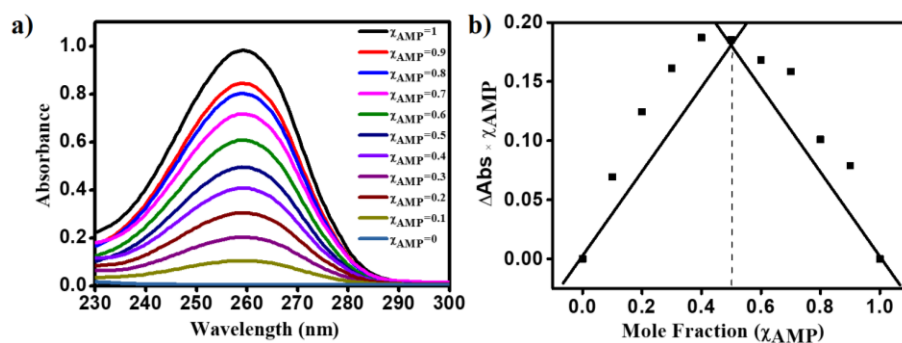


Figure 2.3 a) UV spectrum of AMP upon addition of different concentrations of cobalt chloride. b) Job's plot obtained at its maximum absorption wavelength (260 nm).

2.3.2 Specific gelation with AMP

All the adenine analogues, such as adenine, adenosine, adenosine diphosphate (ADP), and adenosine triphosphate (ATP), were screened for hydrogelation with Co^{2+} .

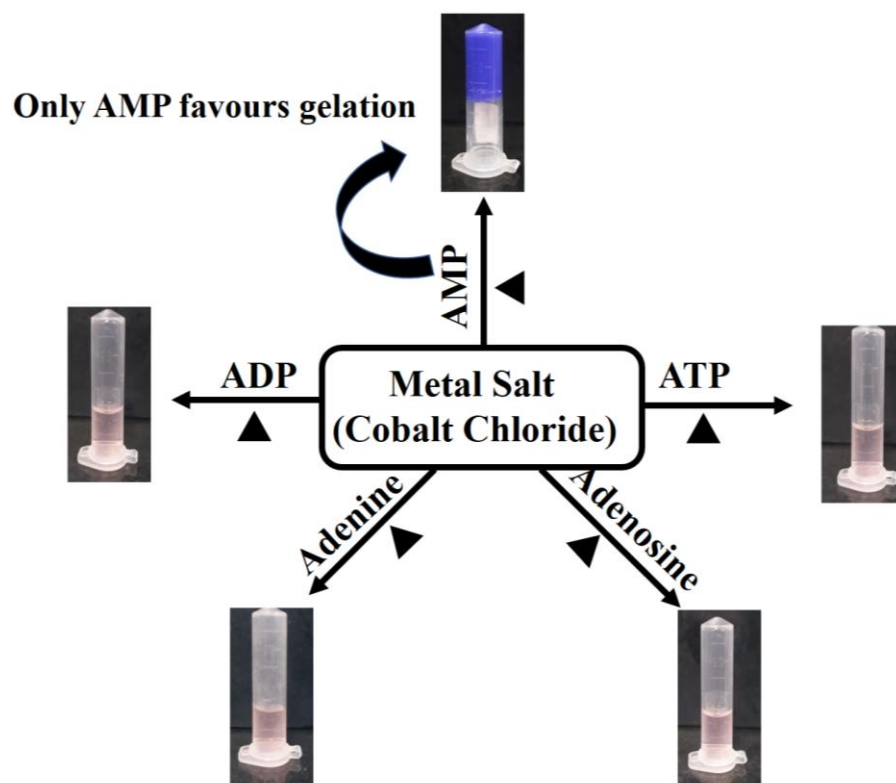


Figure 2.4 Scheme representing the specificity of gel formation only with adenosine 5'-monophosphate over other adenine-based biomolecules.

It is worth mentioning that the gel formation was specific for AMP only, while the other adenine analogues resulted in pink solutions at room

temperature or elevated temperature (60 °C) upon interaction with CoCl_2 at a similar concentration (**Figure 2.4**).

2.3.3 Capability of Co-AMP gel formation in different solvent systems

To investigate the effect of solvent, the gelation was tried in different solvent systems. A stable and robust Co-AMP organogel formed when methanol was used as the solvent. The 1:1 mixture of methanol and water also resulted in hydrogel formation. Pure acetonitrile could not be used due to the restricted solubility of AMP, but a 1:1 mixture of water and acetonitrile resulted in stable gel formation (**Figure 2.5**). The results indicated the versatility of the hydrogel formation in both aqueous and organic media, thereby widening its utility in diverse fields of science.

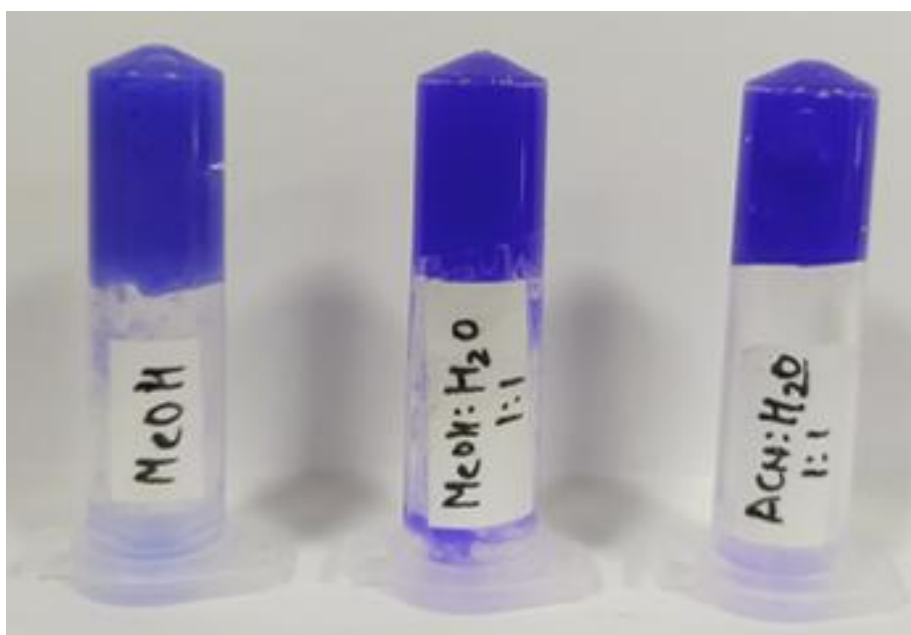


Figure 2.5 Gelation in different solvent media.

2.3.4 Effect of temperature on gelation

The hydrogel formation occurred only when the aqueous mixture of CoCl_2 and AMP was heated above 45 °C. Thus, it is important to investigate the effect of temperature on gelation. For this purpose, the gelation was done at different temperatures from 45 °C to 90 °C. It was observed that the gelation time was reduced significantly from 11

minutes to 50 seconds when the temperature was raised from 45 °C to 90 °C (**Figure 2.6a, 2.6b**). This indicated the crucial role of temperature in the gel formation. This heat-set gel remained stable in the ambient conditions and can be stored for a long time after being formed at an elevated temperature.

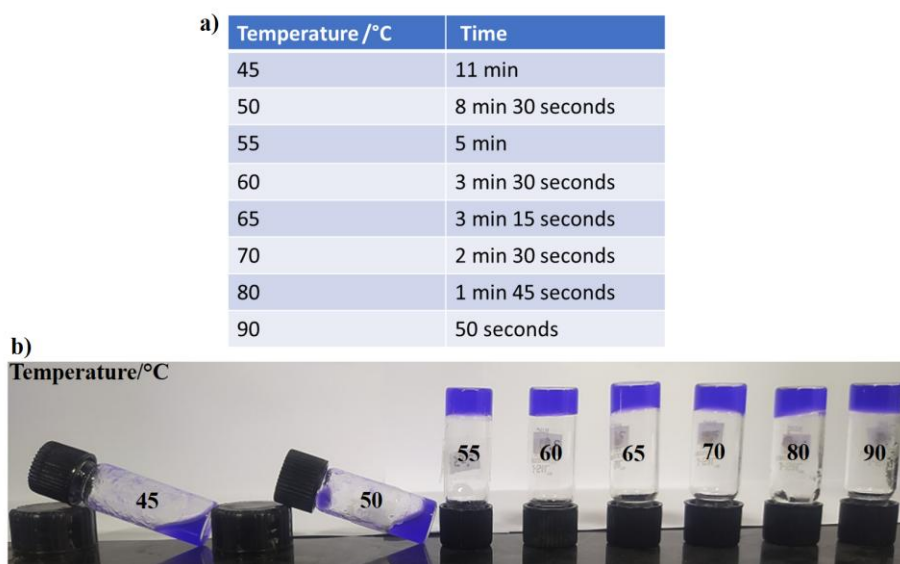


Figure 2.6 a) Table representing gelation time with temperature variation. b) Digital images revealing the gelation tendency with varying temperatures.

2.3.5 pH stability of Co-AMP hydrogel

To investigate the effect of pH on the self-assembled Co-AMP hydrogel, the pH of the reaction medium was varied from pH 1 to 12. Spontaneous hydrogel formation occurred at elevated temperatures in all the cases (**Figure 2.7**). This observation indicated the wide pH stability of the Co-AMP hydrogel. The formed hydrogels were robust and stable at room temperature. The wide pH stability of the hydrogel widens its utilization in electrocatalysis and several other applications.

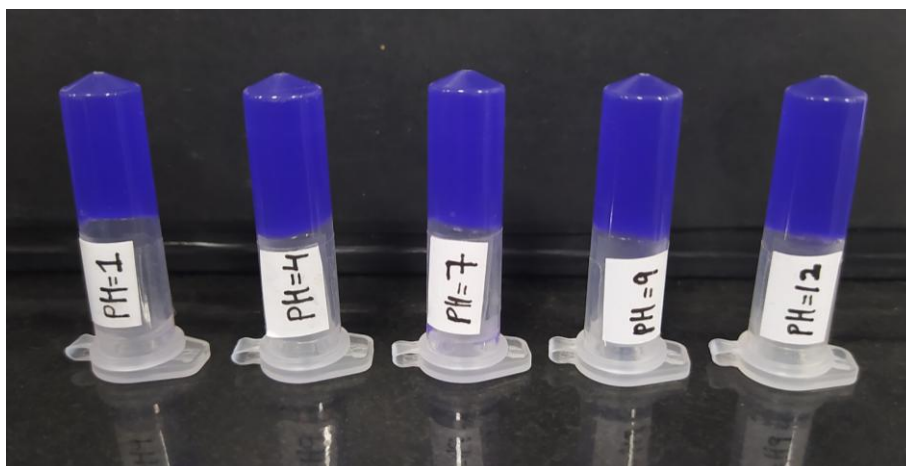


Figure 2.7 Digital image showing wide pH stability of Co-AMP hydrogel.

2.3.6 Chemical stimulus response of Co-AMP hydrogel

Ethylenediaminetetraacetic acid (EDTA) was used as a chemical stimulus to investigate the chemical stimulus responsiveness of the Co-AMP hydrogel. The addition of 0.5 M EDTA solution into the hydrogel resulted in the immediate disintegration of the gel matrix owing to the better chelating ability of Co^{2+} with EDTA (**Figure 2.8**). This observation suggested the crucial role of cobalt in hydrogel formation.

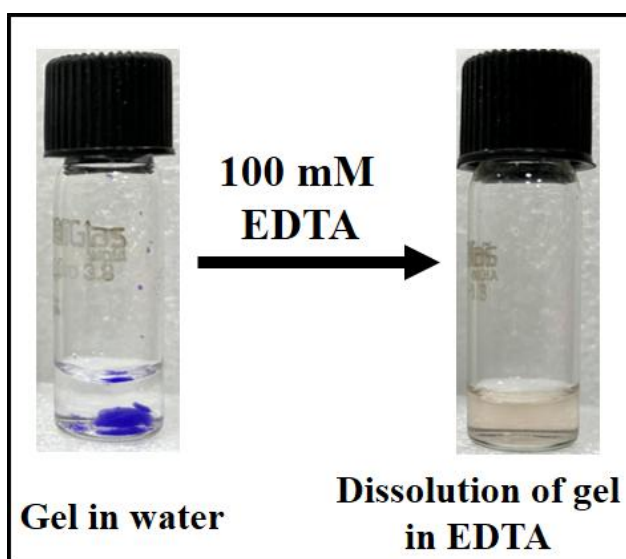


Figure 2.8 Digital image showing the dissolution of CA hydrogel when placed in an aqueous solution of EDTA.

2.4 Characterization of Co-AMP hydrogel

2.4.1 Morphological Characterization

The morphological characterization of Co-AMP hydrogel was done using various microscopic techniques. Field emission scanning electron microscopy (FESEM) images of the hydrogel revealed the formation of intertwined fibers, which entangled into a three-dimensional network of high aspect ratio fibers. The individual fiber had an average diameter of about 50-70 nm and a length of several microns, suggesting that the individual molecular strands were bundled up to form the nanofibers (**Figure 2.9a**). Transmission electron microscopy (TEM) micrographs also corroborated the formation of highly entangled nanofibers with dimensions comparable to those obtained from the SEM images (**Figure 2.9b**). Atomic force microscopy (AFM) images of the hydrogel further confirmed the formation of fibrous nanostructures of Co-AMP with an average height of 4 nm (**Figure 2.9c**).

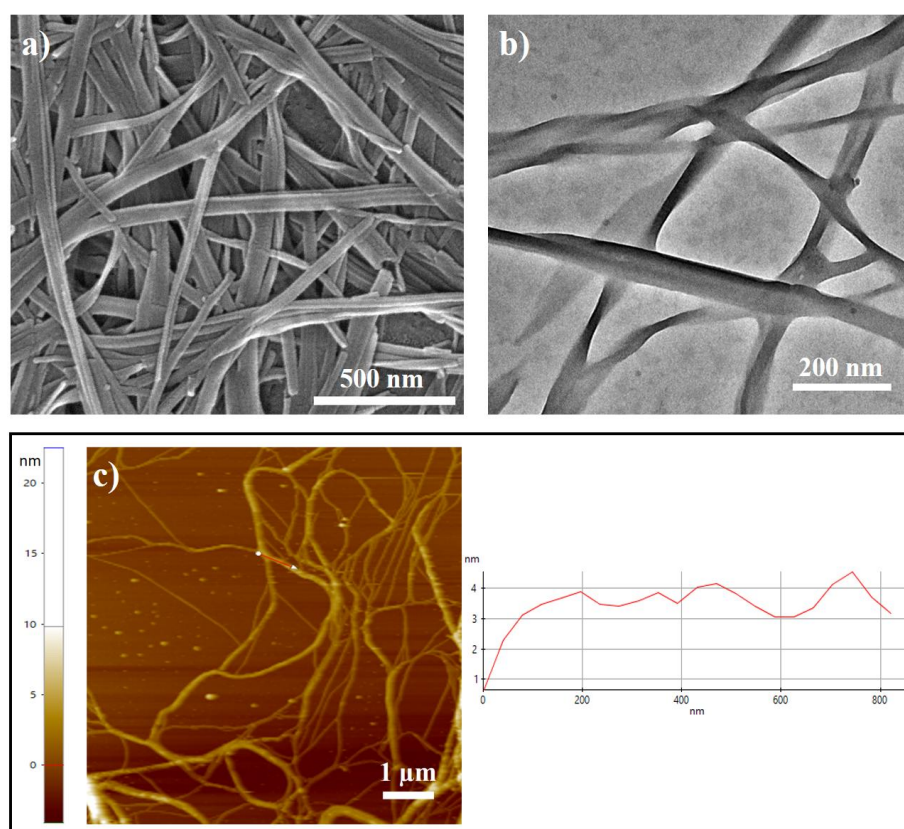


Figure 2.9 a) SEM image of the Co-AMP hydrogel revealing the highly entangled fibrillar morphology. b) TEM image for the Co-AMP hydrogel. c) AFM image ($10 \times 10 \mu\text{m}$) and height profile depicted entangled fibrillar matrix.

2.4.2 Spectroscopic Characterization

Addition of AMP to a CoCl_2 solution at 25 °C showed a pink-colored solution with an absorption peak at 509 nm, indicating the formation of the octahedral (O_h) complex. With an increase in the reaction temperature of the medium, the peak intensified until 45 °C. At 50 °C, the color of the solution turns blue with the appearance of three absorption peaks at around 522, 562, and 638 nm, which are characteristic of tetrahedral cobalt (II) complexes (T_d , ${}^4A_2 \rightarrow {}^4T_1(P)$) [5,6]. The temperature-dependent absorption spectra revealed the transition from O_h to T_d above 45 °C (**Figure 2.10a**). The UV-visible absorption spectra of CoCl_2 , AMP, and Co-AMP hydrogel showed the tetrahedral arrangement around the metal centre in the hydrogel (**Figure 2.10b**).

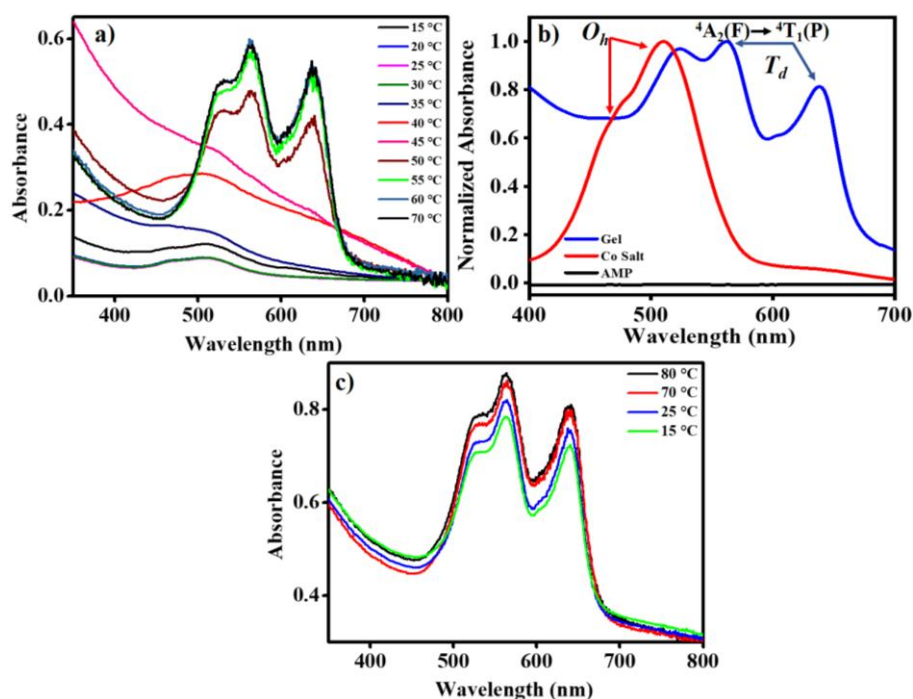


Figure 2.10 a) Temperature-dependent UV-visible spectra from 15 °C to 70 °C, b) UV-visible spectra of CoCl_2 , AMP, and Co-AMP hydrogel, c) UV-visible spectra showing stability of the Co-AMP hydrogel test upon cooling from 80 °C to 15 °C.

To check the stability of the structural transition, the hydrogel was cooled to 15 °C, and no changes in the absorption peaks were observed (**Figure 2.10c**). The results suggested that the hydrogel was formed only when the cobalt metal centre had a tetrahedral arrangement, and a coordination polymer network led to the formation of the

hydrogel. In addition, there was no dissolution of gel and change in the T_d arrangement after the heat-set gel was brought to ambient conditions.

To further understand the interaction between Co^{2+} and AMP within the gel matrix, the FTIR spectrum of the freeze-dried gel was recorded (**Figure 2.11a**). The peaks at 1638 cm^{-1} and 1329 cm^{-1} corresponding to the NH_2 stretching frequency of the base residue in AMP were shifted to 1645 cm^{-1} and 1335 cm^{-1} , respectively, in the Co-AMP gel, indicating coordination of metal with the nitrogen atom [7]. The other peaks at 1329 cm^{-1} and 1253 cm^{-1} show a considerable decrease in intensity, supporting the plausible binding of Co^{2+} with AMP. The peaks at 1089 cm^{-1} and 974 cm^{-1} in AMP are assigned to antisymmetric and symmetric stretching of the PO_3^{2-} , respectively [8]. These peaks were shifted to 1070 cm^{-1} and 982 cm^{-1} , respectively, in the Co-AMP gel, suggesting the interaction of Co^{2+} with the phosphate group. The powder XRD pattern of lyophilized Co-AMP gel shows a distinct peak at $2\theta = 27^\circ$, suggesting the presence of π - π stacking in the matrix with an interlayer separation of 0.32 nm (**Figure 2.11b**). The other sharp peaks signify the high crystallinity of the gel [9].

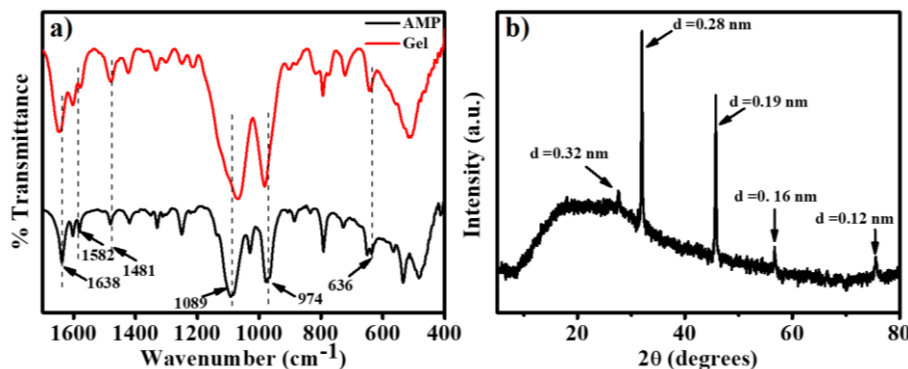


Figure 2.11 a) FTIR spectra of the freeze-dried Co-AMP hydrogel, b) Powder XRD spectrum of Co-AMP hydrogel.

Further, the oxidation state of the cobalt ion and its interactions in the gel were also investigated using X-ray photoelectron spectroscopy (XPS) (**Figure 2.12a**). In the Co 2p spectrum, two peaks corresponding to Co $2p_{1/2}$ and Co $2p_{3/2}$ appeared at 797.28 eV and 781.5 eV respectively along with a spin orbit coupling (Co $2p_{1/2}$ - Co $2p_{3/2}$) value of 15.8 eV

suggesting the +2 oxidation state of cobalt ion in the gel matrix (**Figure 2.12b**) [10]. The deconvoluted O 1s spectrum consists of peaks at binding energies of 536.0 eV, 533.9 eV, and 532.4 eV assigned to OH, P=O, and C-O, respectively. Moreover, the peak at 530.6 eV for Co-O-P linkages revealed the interaction of Co^{2+} with the phosphate group (**Figure 2.12c**) [11]. The deconvoluted N 1s spectrum consists of two peaks at 400 eV and 398.7 eV corresponding to Co-N and pyridinic N, respectively, revealing the coordination of Co^{2+} with the nitrogen atom [12]. These observations were consistent with the FTIR analysis (**Figure 2.12d**). Hence, the plausible binding of Co^{2+} preferably occurs with the N7 position of the nucleobase and the phosphate group to form the coordination polymer network [13].

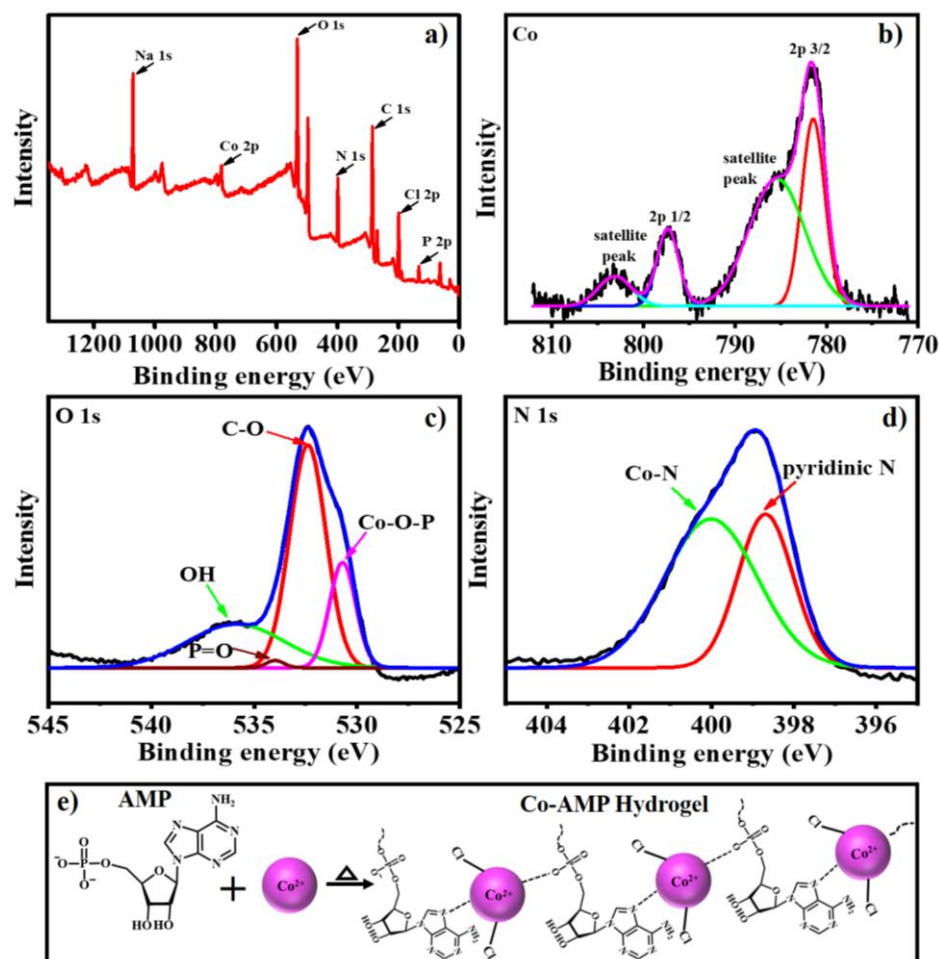


Figure 2.12 a) XPS survey spectrum of Co-AMP hydrogel, b) High-resolution Co 2p XPS spectra, and, c,d) Deconvoluted O 1s and N 1s XPS spectra of Co-AMP hydrogel, e) Plausible structure for Co-AMP hydrogel.

Based on several experimental and microscopic studies, a plausible structure for Co-AMP hydrogel is proposed and depicted in **Figure 2.12e**. The Co^{2+} ions were tetrahedrally coordinated with both the N7 residue of the nucleobase and the phosphate group of AMP to form the coordination polymer network. Further, non-covalent interactions such as hydrogen bonding and π - π stacking between the individual strands facilitated the formation of fibrous entangled nanostructures that could trap water in their matrix.

2.4.3 Mechanical properties of Co-AMP hydrogel

Rheological studies were performed to understand the mechanical behavior and viscoelastic properties of the hydrogel. The strain-sweep rheological study showed that the value of the elastic storage modulus G' was significantly higher than the loss modulus G'' up to a strain of 46%, suggesting the dominance of solid-like elastic behavior over the liquid-like viscous properties. However, G'' dominated over G' beyond a strain of 46%, indicating the conversion of gel to sol state at a strain greater than 46% (**Figure 2.13a**). The dominance of the elastic solid-like behavior over the viscous behavior was further ascertained from a frequency sweep rheological study, which depicted the dominance of G' over G'' by one order of magnitude in the frequency range of 0.1 to 100 rad s^{-1} (**Figure 2.13b**). The thixotropic nature of the Co-AMP hydrogel was studied at room temperature by applying alternating strains of 0.1% and 50% within a prescribed time interval for five successive cycles (**Figure 2.13c**). It was found that initially, when a strain of 0.1% was applied, the value of G' was higher than G'' . However, when the strain was increased to 50%, the gel was converted to sol. The release of strain again led to the domination of the elastic nature of the gel over the viscous nature. Significant recovery in mechanical strength was observed for a strain of 0.1% after five successive cycles, suggesting the potential application of the gel as a soft injectable material.

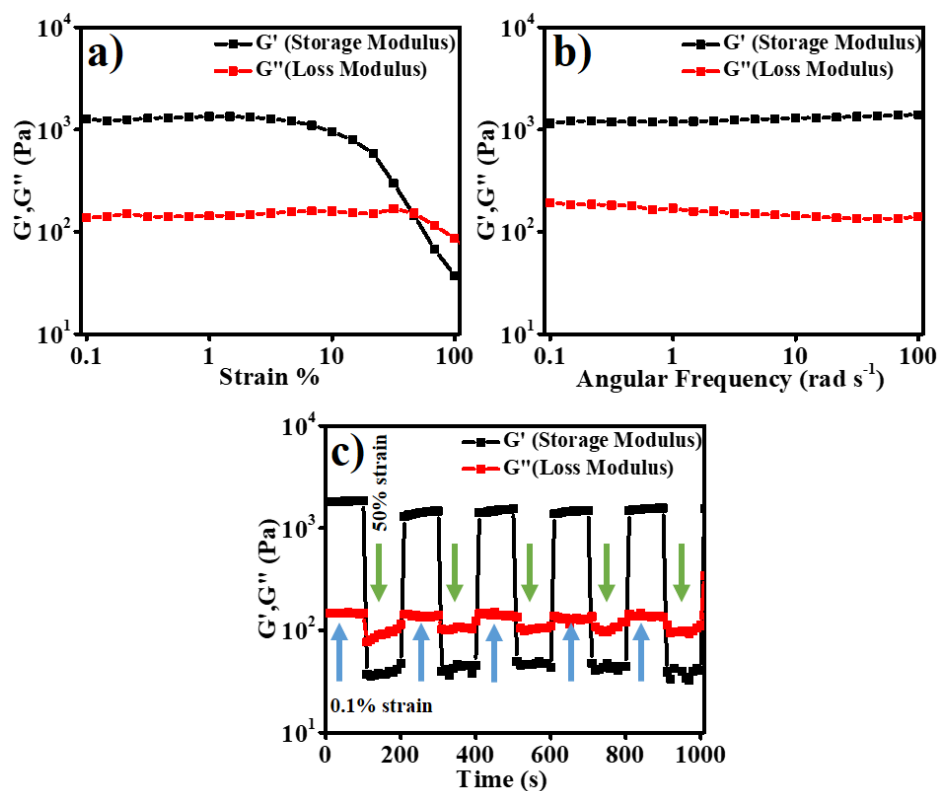


Figure 2.13 a) Dynamic Strain Sweep studies for the Co-AMP hydrogel at a strain of 1%, b) Frequency sweep studies for the Co-AMP hydrogel, c) Thixotropic analysis for the Co-AMP hydrogel.

2.4.4 Magnetic properties of Co-AMP hydrogel

The magnetic behavior of Co-AMP gel was studied by field and temperature-dependent magnetic measurements (**Figure 2.14a**). The isothermal magnetization curve was recorded at three temperatures: 5 K, 150 K, and 300 K. At 5 K, an S-shaped (M-H) curve suggested the superparamagnetic behavior of the hydrogel. The saturation magnetization value (M_s) was found to be 13.9 emu/g and increases at a lower temperature. The remanent magnetization (M_r) and coercivity (H_c) values were found to be 0.01 emu/g and 35.3 Oe, respectively (**Figure 2.14b**). At higher temperatures, the hydrogel exhibits paramagnetic behavior. The M-T curve was recorded in FC (Field Cooled) modes and ZFC (Zero Field Cooled).

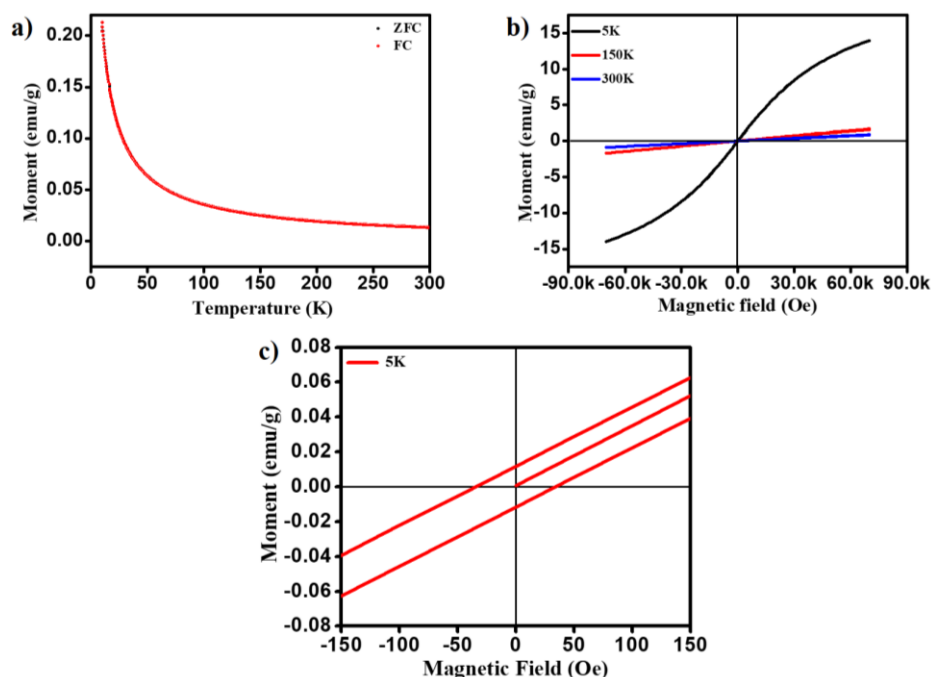


Figure 2.14 a) Temperature-dependent magnetization curve for Co-AMP gel, b) Isothermal magnetization curve at three different temperatures, 5 K, 150 K, and 300 K, c) Magnified magnetic hysteresis curve for Co-AMP gel at 5 K.

At room temperature, the M-H curve shows a linear behaviour that typically reflects paramagnetic behaviour of the material. When the temperature is lowered, the magnetic moment continues to increase and does not saturate even at a temperature as low as 5 K, which suggests the superparamagnetic behavior of the Co-AMP hydrogel (**Figure 2.14c**).

2.5 Long-term stability of Co-AMP hydrogel in ambient conditions

The hydrogel was highly stable and could be stored for a long period at room temperature. No visual changes were observed in the hydrogel even after standing for more than six months at room temperature, and its viscoelastic properties were retained as evident from the strain sweep experiment. No change in the absorption peaks in the UV-visible spectrum also confirmed the presence of tetrahedrally stabilised Co^{2+} ions in Co-AMP hydrogel even after aging for several months (**Figure 2.15**).

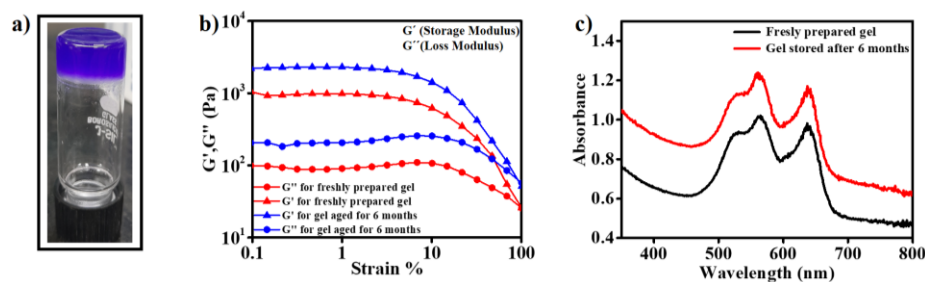


Figure 2.15 a) Digital image showing the stability of Co-AMP hydrogel aged for 6 months, b) Dynamic Strain Sweep studies for freshly prepared and aged Co-AMP hydrogels, c) UV-visible spectra for Co-AMP hydrogel stored for 6 months and freshly prepared hydrogels.

2.6 Conclusion

In conclusion, a very facile and straightforward approach was utilized to fabricate a supramolecular self-assembled cobalt-based hydrogel. The hydrogel was formed by the simple mixing of aqueous solutions of cobalt and AMP, followed by heating at 60 °C. This methodology is devoid of any chemical modification, thereby preserving the native form of precursor biomolecules in the functional material. The tetrahedrally coordinated blue-colored Co-AMP hydrogel was highly stable at room temperature and within a wide pH window ranging from 1 to 12. The gel was mechanically robust and thixotropic. The hydrogel can be stored for a long period in ambient conditions. The ease of fabrication and the exceptional stability of Co-AMP hydrogel widen its utility in diverse fields of science.

2.7 Experimental Section

2.7.1 Materials

Adenosine 5'-monophosphate disodium salt (AMP- Na_2), Adenosine 5'-diphosphate disodium salt (ADP- Na_2), Adenosine 5'-diphosphate trisodium salt (ATP- Na_2), Adenine, Adenosine, Cobalt Chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), Ethylenediaminetetraacetic acid disodium salt dihydrate, and Water HPLC grade were purchased from Sisco Research Laboratory, India (SRL). Methanol and acetonitrile were

procured from Spectrochem. All chemicals were used as received without further purification.

2.7.2 Instrumentation

UV-visible studies were performed using a Perkin-Elmer Lambda 750 spectrophotometer at room temperature. The microscopic images of the hydrogel were obtained using a Nova Nano FE-SEM instrument. The sample was drop cast and coated with Au. TEM (Transmission Electron Microscopy) measurements were performed using 300 kV FEI Tecnai G2, F30 instrument. AFM experiments were carried out using an NX-10 PARK system in a tapping mode, by depositing the diluted hydrogel on mica. The powder X-ray diffraction pattern for freeze-dried gel was recorded on a Rigaku Smartlab instrument having a Cu K α source with 0.154 nm X-ray wavelength. FTIR studies were performed using a Bruker ALPHA 11 spectrometer. Rheological measurements of the fabricated hydrogels were recorded on an Anton Paar Physica MCR 301 rheometer at room temperature. The dynamic strain sweep measurements were carried out at a constant angular frequency of 10 rad s⁻¹ and frequency sweep experiments were measured at a 5 % constant strain value. The thixotropic loop test studies were performed by changing the strain value from 1 % to 50 % at a constant 10 rad s⁻¹ of angular frequency for every time interval.

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Chapter 3

Multifunctional Co-AMP Hydrogel for Versatile Applications

This chapter highlights the versatile applications of Co-AMP hydrogel which is discussed in Chapter 2 in detail. It emphasizes the multiutility of a single matrix and the potential of AMP-based hydrogels for a broad range of applications. This Chapter is subdivided into three chapters, where each chapter describes the specific application of the hydrogel.

- **Chapter 3A** includes the utilization of Co-AMP hydrogel as a pH-dependent catalase and peroxidase mimicking system. It is used to mitigate the cellular oxidative stress due to H_2O_2 owing to its catalase-mimicking activity.
- **Chapter 3B** shows the ability of Co-AMP hydrogel to mimic the carbonic anhydrase enzyme. This is the first report where non-zinc and non-histidine system is being exploited as a CA mimic
- **Chapter 3C** explores the electrocatalytic activity of Co-AMP hydrogel. It is used as a bifunctional electrocatalyst for alkaline water electrolysis. The high catalytic efficiency and exceptional stability of the Co-AMP hydrogel demonstrate its applicability in industrial conditions.

Chapter 3A

Cobalt-Adenosine Monophosphate (Co-AMP) Supramolecular Hydrogel with pH-responsive Multi-Nanozymatic Activity

3A.1 Introduction

Enzymes are responsible for diverse reactions in the metabolism that include the prevention of various diseases. High concentrations of H_2O_2 can be detrimental to cells, leading to cell apoptosis and delayed wound healing, as it prevents connective tissue formation. Therefore, reducing H_2O_2 levels to form O_2 through enzymatic processes is critical for reducing oxidative stress, preventing infection, and promoting tissue formation [1,2]. In natural systems, this process of catalytic conversion from H_2O_2 to H_2O and O_2 is achieved by the catalase enzyme. However, practical applications of natural enzymes have limitations owing to their high production cost, short lifetime, and high sensitivity of their catalytic activity towards reaction conditions such as changes in pH or temperature [3,4].

Consequently, significant research efforts have been directed toward developing artificial enzyme mimics that can effectively imitate their catalytic functions. Different kinds of inorganic nanoparticles, metal complexes, and metal-organic supramolecular materials have been developed as enzyme mimetic systems with higher stability and better activity. For mimicking catalase-like activity, various systems such as CeNPs, Co_3O_4 NPs, di-copper peptidyl complex, etc. have been developed [2,5-7]. For better processability and to provide a cell or tissue-like environment, the active enzyme mimetic systems are often loaded into hydrogels or nanofibrous membranes [8]. However, the inherent enzyme mimetic behavior of a self-assembled metallogel is of great interest for diverse applications. In this context, nucleotide-based

hydrogel can serve as the best template owing to its biocompatibility, hydrated matrix.

Herein, the Co-AMP hydrogel is exploited as a pH-dependent multienzyme mimicking system. It can mimic catalase and peroxidase activity under different pH conditions. The hydrogel showed remarkable pH and thermal stability. The binding of AMP with Co^{2+} significantly reduces the cytotoxicity of the metal ion. Interestingly, the metallogel exhibits catalase-mimicking activity under basic conditions, whereas under acidic conditions, the same metallogel demonstrates peroxidase-like activity. The multi-enzymatic behavior of the metallogel was further demonstrated for phenoxazinone synthase activity. In vitro cell studies revealed the potential of the Co-AMP hydrogel in reducing oxidative stress by scavenging reactive oxygen species generated by the presence of excessive H_2O_2 . It also revealed the potent antioxidant behaviour of the hydrogel. The simple synthetic protocol, high stability, and multienzymatic activity make the hydrogel useful for sensing and other biomedical applications.

3A.2 Results and Discussion

A simple mixing of aqueous solutions of CoCl_2 and AMP, followed by heating the mixture at $60\text{ }^\circ\text{C}$, led to the spontaneous formation of a blue-colored stable hydrogel. The Co-AMP hydrogel (1:1) was characterized using several spectroscopic and microscopic techniques already discussed in **Chapter 2** in detail.

3A.2.1 Catalase-like activity of Co-AMP hydrogel

Catalase (CAT) is among the most important oxidoreductases found in the human body and plays an essential role in the removal of reactive oxygen species, thus preventing damage to the cell membrane. We explored the Co-AMP xerogel as a CAT mimic by estimating the O_2 produced from the decomposition of H_2O_2 . The addition of Co-AMP xerogel to an aqueous solution of H_2O_2 (50 mM) at near-neutral pH (pH 7.4) led to the formation of bubbles, observable to the naked eye. The

results suggested the production of O_2 by virtue of the CAT-mimicking activity of Co-AMP (Figure 3A.1a). The catalase activity of Co-AMP xerogel was further ascertained spectrophotometrically using Europium-tetracycline (Eu-Tc) fluorescence assay. Eu^{3+} ions are known to bind to tetracycline to form an Eu-Tc complex, which is weakly fluorescent (Figure 3A.1b). The addition of H_2O_2 resulted in an increase in the fluorescence intensity at 615 nm due to the formation of a peroxo-complex $[Eu(HP)(Tc)]$ [9].

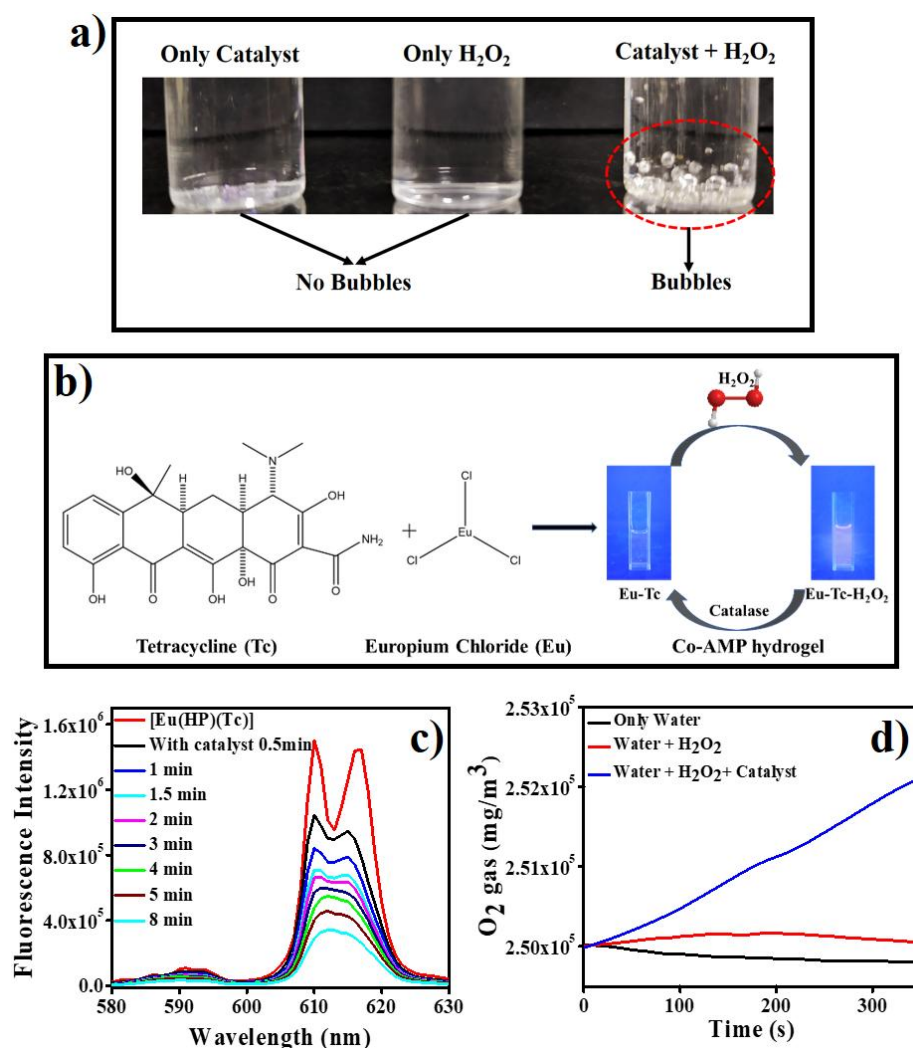


Figure 3A.1 a) Digital image showing the generations of O_2 bubbles during the catalase activity in the presence of H_2O_2 and Co-AMP hydrogel at pH 7.4, b) Synthetic scheme for the fluorescent Eu-Tc- H_2O_2 complex and its fluorescence quenching upon addition of catalase enzyme or catalase mimics, c) Fluorescence spectra depicting the quenching of fluorescence intensity of $[Eu(HP)(Tc)]$ complex upon addition of Co-AMP hydrogel, d) Generation of O_2 gas in the presence of catalyst detected by O_2 sensor.

Upon degradation of H_2O_2 , the fluorescence of the $[\text{Eu}(\text{HP})(\text{Tc})]$ complex at 615 nm is quenched (**Figure 3A.1c**). Therefore, the progress of the H_2O_2 degradation by CAT and the simultaneous formation of O_2 can be monitored using fluorescence spectroscopy. The addition of Co-AMP (0.5 mg/mL) to the $[\text{Eu}(\text{HP})(\text{Tc})]$ complex resulted in a decrease in fluorescence intensity at 615 nm started decreasing, suggesting the formation of O_2 . Quantitative estimation of the O_2 produced by the decomposition of H_2O_2 in the presence of Co-AMP xerogel was studied using an oxygen gas sensor. Control experiments showed that neither H_2O_2 nor Co-AMP xerogel alone was capable of producing O_2 gas (**Figure 3A.1d**). The CAT-like activity of the Co-AMP xerogel was performed at varying pH (2-12). Under acidic conditions, negligible bubble formation was observed suggesting that Co-AMP xerogel does not show CAT-mimicking activity under these conditions. However, under basic conditions, the O_2 bubble formation in the solution was observed. The intensity of O_2 bubble formation increases with the increase in the basicity of the medium [10].

The results were further verified by quantitative estimation of the produced O_2 gas using an O_2 gas sensor (**Figure 3A.2a**). Further, the variation of catalyst amount from 0.05 mg/mL to 1.0 mg/mL increased O_2 generation, suggesting faster degradation of H_2O_2 (**Figure 3A.2b**). The stability of the catalyst was studied by comparing the activity of freshly prepared gel and after ageing (**Figure 3A.2c**). Steady-state kinetics were performed at pH 7.4 to calculate the kinetic parameters by varying the concentration of H_2O_2 (5 mM-150 mM) (**Figure 3A.2d**). The reaction followed Michaelis-Menten kinetics. The Michaelis constant (K_m) and maximum velocity ($V_{\max}$) were calculated to be 57.35 mM and 40 mM/s, respectively.

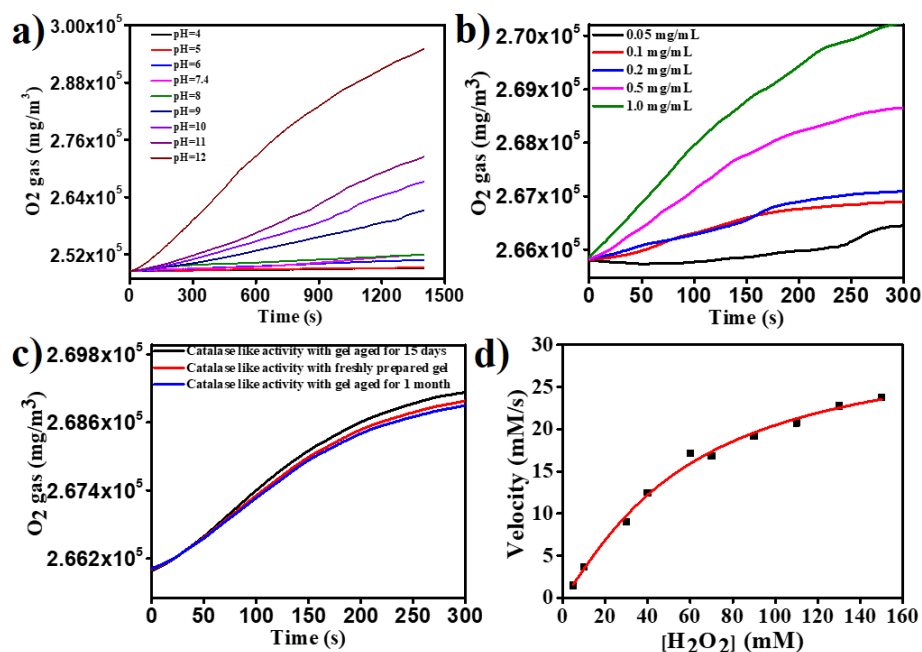


Figure 3A.2 a) pH-dependent study for catalase mimicking activity from pH 4 to 12, b) Generation of O₂ with variable concentration of Co-AMP hydrogel ranging from 0.05 mg/mL to 1.0 mg/mL, c) Catalase-like activity with freshly prepared hydrogel and after storing the gel for 15 days and 1 month, d) Steady-state kinetics assay for catalase activity, with respect to the concentration of H₂O₂.

3A.2.2 Peroxidase-like activity of Co-AMP hydrogel

The pH-dependent catalase activity of Co-AMP xerogel prompted us to study the peroxidase activity as pH-switchable peroxidase-catalase activities are demonstrated by several enzyme mimics [6,10-13]. To evaluate the peroxidase-like activity of the Co-AMP gel, 3,3',5,5'-tetramethylbenzidine (TMB) was used as a chromogenic substrate. TMB in its native form is colorless, however, upon oxidation, it gives an intense blue color with an absorption maximum at 652 nm. As expected, the Co-AMP gel showed peroxidase-like activity only under acidic conditions (**Figure 3A.3a**). Upon incubating TMB (1 mM) with H₂O₂ (5 mM) and Co-AMP xerogel (0.5 mg/mL) in acetate buffer (0.1M, pH 4.0), an observable change from colorless to blue was observed. The UV-visible spectrum showed the appearance of a characteristic peak of oxidized TMB at 652 nm, which intensified with time, indicating the peroxidase mimetic behavior of Co-AMP gel (**Figure 3A.3b**).

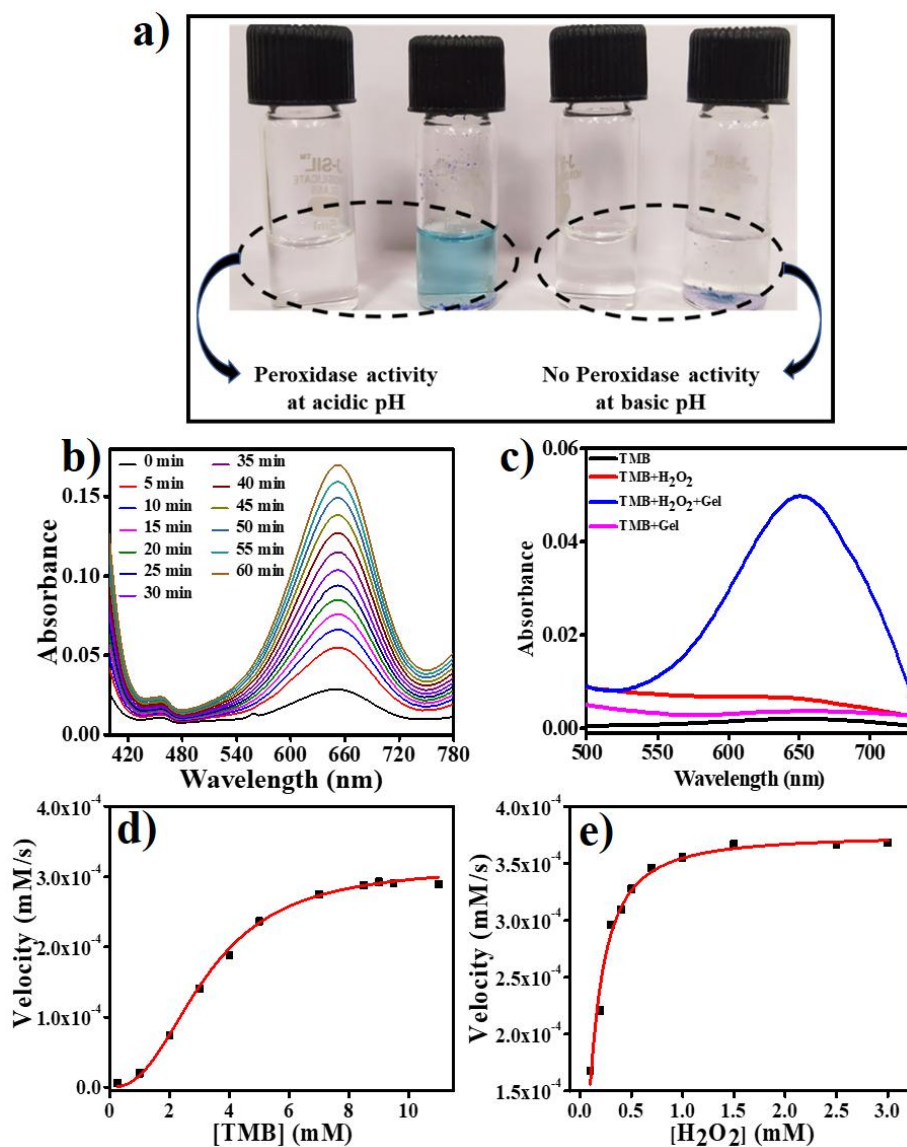


Figure 3A.3 a) Digital image depicting the pH-dependent peroxidase mimicking activity of Co-AMP hydrogel in the presence of H₂O₂ and TMB, b) Time-dependent TMB oxidation for the peroxidase mimicking activity of Co-AMP gel, c) UV-Visible spectra showing the oxidation of TMB in various conditions: i) in presence of H₂O₂ and Co-AMP gel, (ii) in presence of H₂O₂, (iii) in presence of Co-AMP gel, and (iv) Only TMB, d) Steady-state kinetics assay, for peroxidase activity with respect to the concentration of TMB, e) Steady-state kinetics assay, for peroxidase activity with respect to the concentration of H₂O₂.

Control experiments were performed in the absence of the catalyst which showed that reaction did not proceed in absence of either the reactants or the catalyst (**Figure 3A.3c**). The kinetic parameters for the peroxidase activity of Co-AMP gel were evaluated by steady-state kinetics, wherein the concentration of one of the reactants (TMB or

H₂O₂) was kept constant and the concentration of the other reactant was varied. The results indicated that the reaction followed a typical Michaelis-Menten kinetics for both substrates. The K_m and V_{max} with H₂O₂ were 0.12 mM and 3.74×10^{-4} mM/s, respectively (**Figure 3A.3d**). The obtained kinetic parameters (K_m and V_{max}) for TMB were 3.21 mM and 3.14×10^{-4} mM/s, respectively (**Figure 3A.3e**). The K_m values obtained by varying the H₂O₂ concentration were much lower than that of natural Horseradish Peroxidase (HRP), suggesting that Co-AMP has a higher affinity for H₂O₂ as compared to the natural HRP.

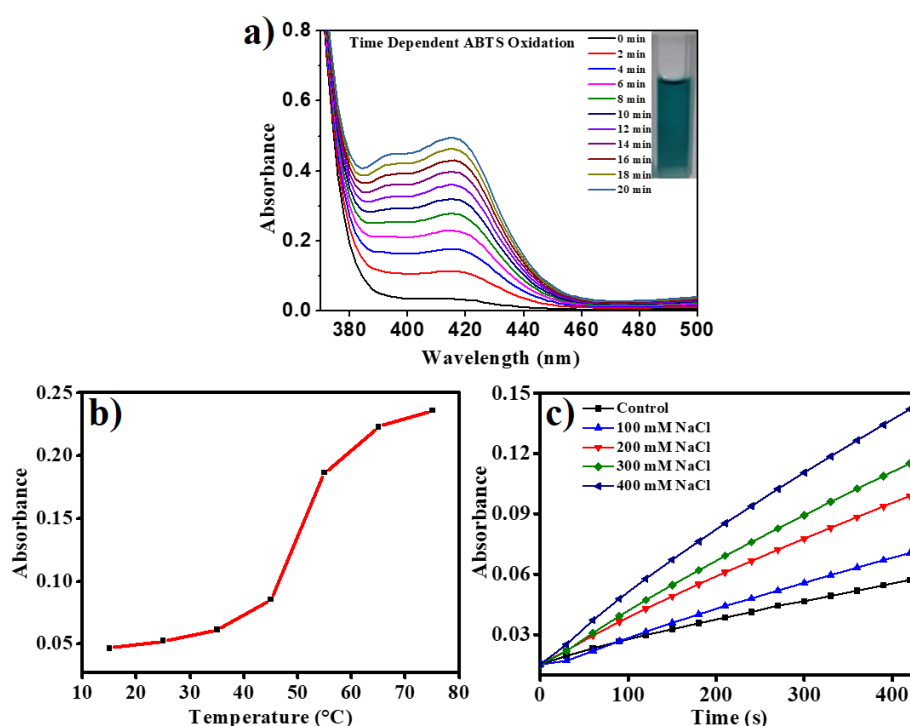


Figure 3A.4 a) UV-visible spectra depicting time-dependent oxidation of ABTS in the presence of H₂O₂ by Co-AMP hydrogel, b) Effect of temperature on the peroxidase activity using Co-AMP hydrogel as the catalyst, c) Effect of ionic strength on the peroxidase activity using Co-AMP hydrogel as the catalyst.

Co-AMP hydrogel was also capable of oxidizing 2,2'-azino bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), which is a chromogenic substrate to demonstrate the peroxidase-like activity. The colorless substrate changed to green color upon oxidation, which is further confirmed spectrophotometrically by recording its absorbance at 416 nm. The increase in absorbance with time indicated the peroxidase

mimicking activity of the hydrogel (**Figure 3A.4a**). To evaluate the stability of the Co-AMP xerogel under variable reaction conditions, we performed the peroxidase activity studies at elevated temperature and increased ionic strength. the catalytic activity of the Co-AMP xerogel was enhanced with an increase in temperature. From the UV-visible studies of oxidized TMB, it was observed that the peroxidase activity was enhanced nearly five times when the temperature was increased from 25 °C to 75 °C (**Figure 3A.4b**). The ionic strength of the medium was varied with a salt concentration from 0 to 400 mM. It was observed that the catalytic activity increased with increasing salt concentration (**Figure 3A.4c**).

The results show the stability and enzymatic activity of Co-AMP xerogel under harsh reaction conditions where the natural enzymes get denatured. The Co-AMP xerogel showed peroxidase-mimicking activity only under acidic conditions ($\text{pH} < 7.0$). Under alkaline or neutral pH, no peroxidase activity was observed, which can be attributed to the decomposition of H_2O_2 under these conditions.

3A.2.3 Mechanism of Peroxidase-like Activity

Previous literature reports suggest that the peroxidase-like activity of nanozymes proceeds either via $\cdot\text{OH}$ radical generation or an electron transfer process [7]. To confirm the mechanism, terephthalic acid was used as a fluorescent probe. Terephthalic acid exhibits minimal intrinsic fluorescence; however, the 2-hydroxy-terephthalic acid generated by its reaction with $\cdot\text{OH}$ radicals shows intense blue fluorescence with an emission maximum at 440 nm. Surprisingly, the fluorescence intensity at 440 nm of the mixture containing terephthalic acid, H_2O_2 , and Co-AMP xerogel got quenched with time, suggesting that the catalyst acted as a $\cdot\text{OH}$ radical scavenger, rather than producing them (**Figure 3A.5a**).

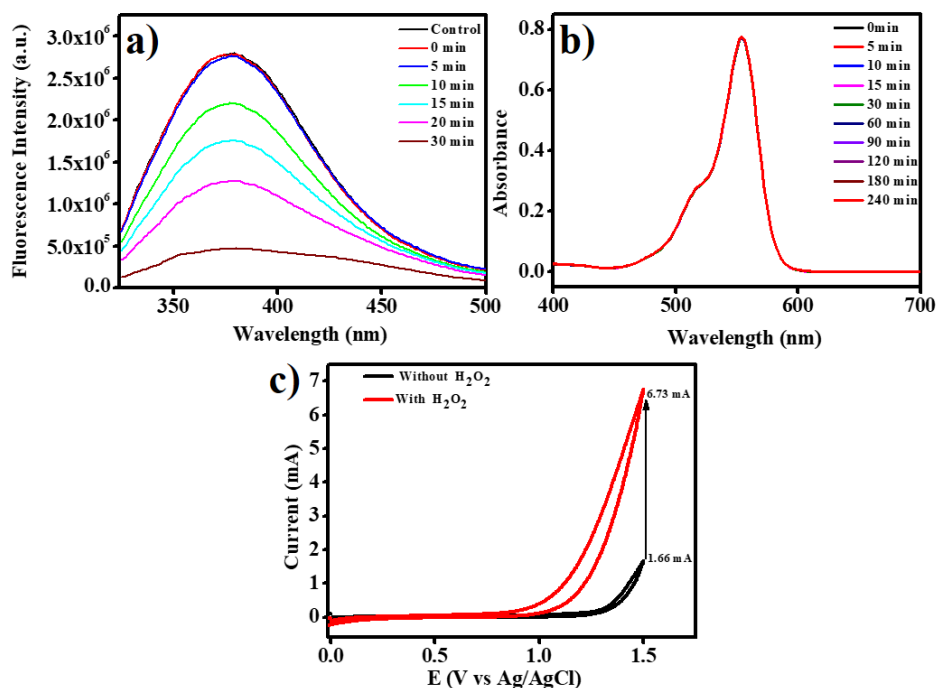


Figure 3A.5 a) Decrease in fluorescence intensity with time using Terephthalic acid (TA) as a fluorescent probe in the presence of Co-AMP gel. b) UV-visible spectra: RhB + H₂O₂ + Co-AMP gel from 0 min to 2 hours. c) Cyclic Voltammetry curve of Co-AMP xerogel deposited on carbon cloth in 100 mM sodium acetate buffer (pH 4.0).

To further confirm the non-involvement of $\cdot\text{OH}$ radicals in the system, RhB dye was incubated with a mixture of H₂O₂ and Co-AMP gel. UV-visible studies show no change in absorbance at 554 nm, which confirms that the reaction did not proceed through the $\cdot\text{OH}$ radical pathway (**Figure 3A.5b**) [14]. To understand the involvement of the electron transfer process of the enzymatic activity, cyclic voltammetry studies were carried out. A carbon cloth was modified with Co-AMP xerogel, and the reduction current response was measured in the absence and presence of H₂O₂ (20 mM). The current increases considerably in the presence of H₂O₂ with a peak at 1.5 V vs Ag/AgCl in acetate buffer (pH 4.0), whereas no current change was observed in the absence of H₂O₂ (**Figure 3A.5c**). The results suggest that the peroxidase activity of Co-AMP xerogel proceeds via an electron transfer pathway as reported for other nanomaterials [14-16].

3.2.4 Phenoxazinone synthase-like activity of Co-AMP hydrogel

Phenoxazinone synthase (PHS) is an oligomeric pentanuclear copper-containing oxidase enzyme found in the bacterium *Streptomyces antibioticus* that uses molecular oxygen for oxidation. It is utilized in the final step of the synthesis of actinomycin D, where it catalyzes the oxidative coupling of two molecules of a substituted 2-aminophenol to form the phenoxazinone chromophore [17]. The PHS mimicking activity of the Co-AMP xerogel was studied using *o*-aminophenol (OAP) as a substrate in methanol under ambient conditions. **Figure 3A.6a** shows the time-dependent UV-visible studies of the oxidation of OAP (0.01M) to 2-aminophenoxazin-3-one using Co-AMP xerogel (1 mg/mL) as a catalyst. As evident from the figure, a steady increase in the absorbance of the band at 430 nm was observed with an increase in time, suggesting the gradual conversion of OAP to 2-aminophenoxazin-3-one. The control experiment in the absence of the catalyst shows a negligible increase in the absorption. Detailed kinetic studies were performed by varying the concentration of OAP from 0.005 M to 0.011 M and monitoring the absorbance values at 430 nm for 30 minutes at an interval of 5 minutes for each set. The rate was determined by linear regression from the slope of absorbance vs. time. The kinetic parameters were evaluated using a Michaelis-Menten model, and the K_m and V_{max} values were calculated to be 0.005 M and 0.016 M/min, respectively (**Figure 3A.6b**). ESI-mass spectrometric study of the product obtained after the addition of Co-AMP xerogel to the methanolic OAP solution confirmed the formation of 2-aminophenoxazin-3-one through the presence of a strong peak at $m/z = 213.2124$ (**Figure 3A.6c**).

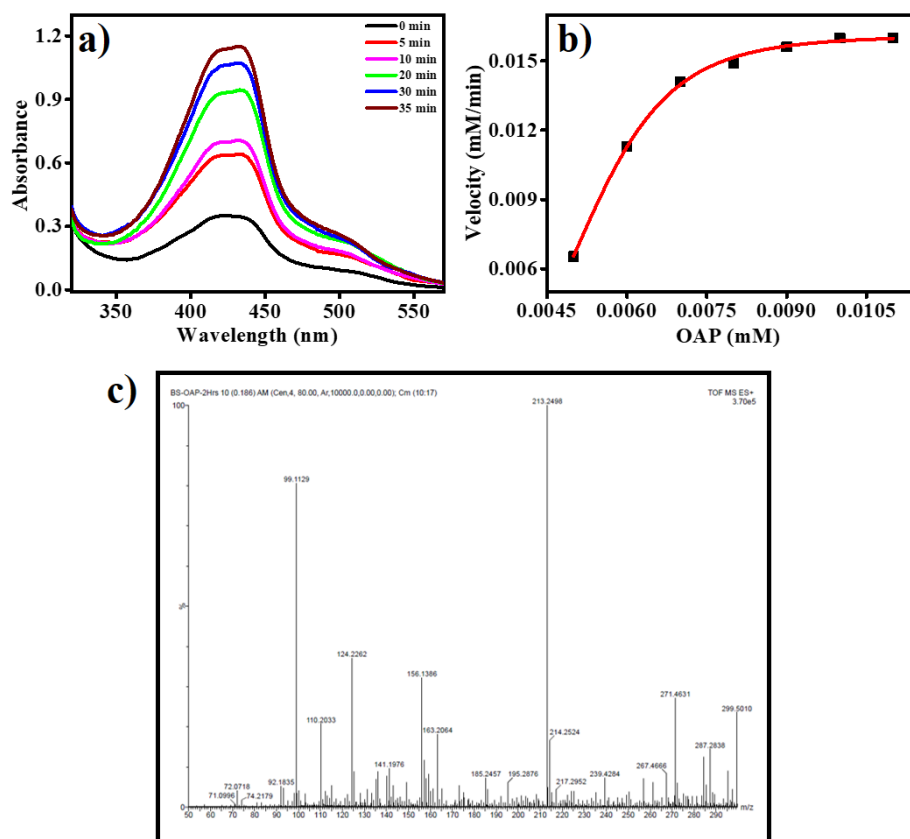


Figure 3A.6 a) Time-dependent *o*-aminophenol oxidation indicating phenoxazinone mimicking activity of Co-AMP gel, b) Steady-state kinetics assay using 1mg/mL Co-AMP gel with respect to the concentration of *o*-aminophenol, c) ESI-MS spectrum of 2-aminophenoxazin-3-one.

3A.2.5 Glutathione sensing

The peroxidase mimetic activity of the Co-AMP gel was utilized for the optical detection of an important tripeptide, glutathione (GSH). GSH, by virtue of the presence of the thiol group, is reducing in nature and can reduce the oxidized TMB (blue) back to TMB (colorless) [18]. Therefore, the peroxidase-like activity of the Co-AMP gel could be utilized for the detection of GSH. The addition of GSH to the oxidized TMB resulted in a decrease in the absorbance at 652 nm corresponding to oxidized TMB, and with an increase in concentration of GSH, the solution turned colorless. The color change was a result of the reduction of oxidized TMB (TMB^{++}) to TMB. To check the sensitivity, the concentration of GSH was varied in the range of 0-120 μM (Figure 3A.7a). The linear relationship was observed between the change in

absorbance and concentration of GSH up to 70 μM (**Figure 3A.7b**). The limit of detection (LOD) was calculated to be 0.075 μM using the formula ($\text{LOD} = 3\sigma/s$), where σ is the standard deviation and s refers to the slope. Further, the aging of hydrogel did not have any significant effect on the GSH sensing behaviour as results were consistent for both the aged and fresh gels (**Figure 3A.7c**).

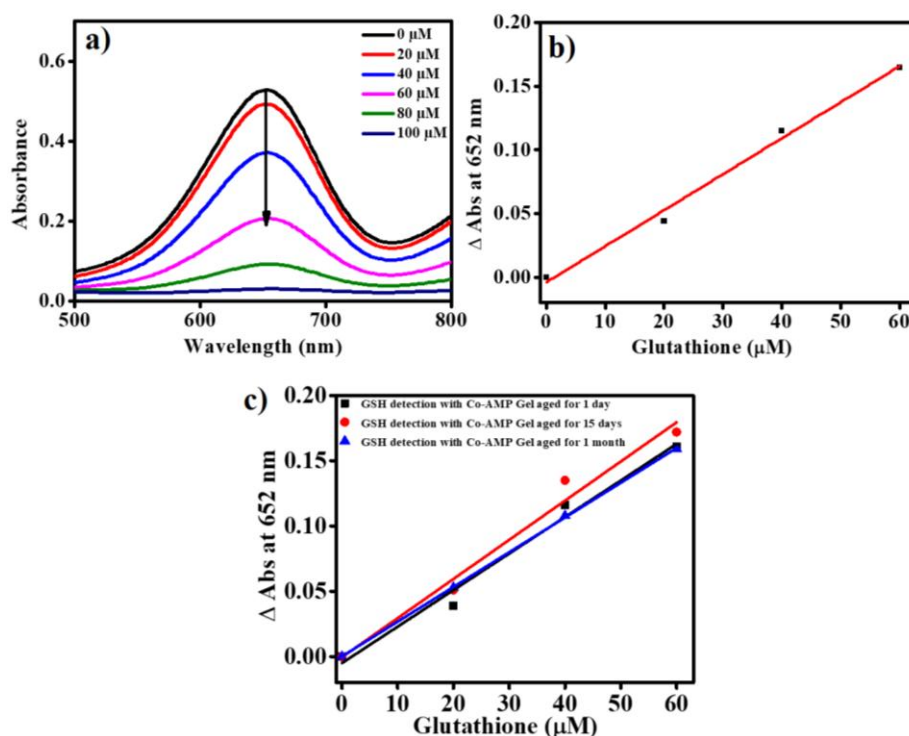


Figure 3A.7 a) UV- visible studies showing the detection of glutathione by Co-AMP hydrogel as indicated by a decrease in absorbance value at 652 nm, b) Linear change observed for glutathione detection in the range of 0-70 μM , c) Linear change observed for glutathione detection in the range of 0-70 μM with fresh hydrogel and gels aged for 15 days and 1 month.

3A.2.6 Co-AMP hydrogel-driven H_2O_2 depletion for the protection of mammalian cells

In vitro studies were performed, leveraging the catalase-mimicking activity of Co-AMP hydrogel to deplete H_2O_2 and decrease oxidative stress in the cells. Oxidative stress was induced in human stomach cells (AGS) by exposing them to different concentrations of

H₂O₂ (100–300 μ M). The cell viability of AGS cells was significantly decreased with increasing H₂O₂ concentration ($p < 0.05$, $p < 0.01$).

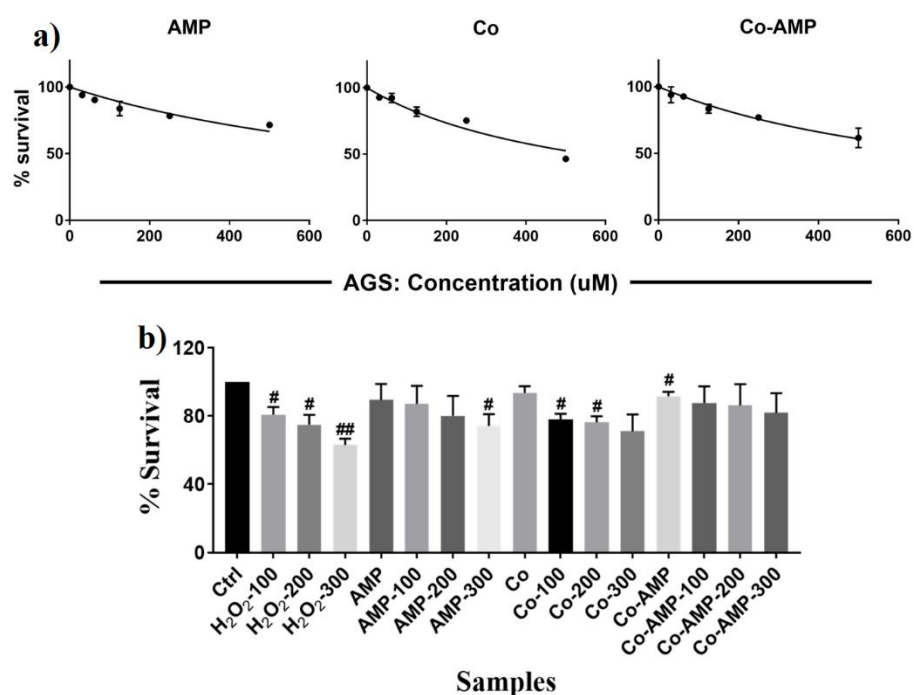


Figure 3A.8 Analysis of cellular viability of AGS cells in the presence and absence of AMP, Co, Co-AMP, and H₂O₂: a) Cellular viability after exposure to different concentrations of compounds (I) AMP, (II) Co, and (III) Co-AMP, b) Viability in cells pre-treated with AMP, Co, and Co-AMP, followed by H₂O₂ exposure. Data were statistically analyzed using an unpaired *t*-test. *p* values were estimated using GraphPad Prism version 7, and *p* values of 0.05, 0.01, and 0.001 were considered statistically significant and represented by */#, **/##, and ***/###, denoting upregulation/downregulation, respectively.

Firstly, a range of concentrations of AMP (30–500 μ M), Co (30–500 μ M), and Co-AMP (30–500 μ M) were tested for inducing any potential cell death in AGS cells (Figure S24A). The IC₅₀ values of AMP, Co, and Co-AMP were found to be 1002 μ M, 553.2 μ M, and 780 μ M, respectively. Next, to understand the protective effect of Co-AMP hydrogel against oxidative stress induced by H₂O₂, AGS cells were pre-treated with 156 μ M Co-AMP hydrogel for 48 hours, followed by the treatment with 100, 200, and 300 μ M H₂O₂ for 2 hours. In the absence of Co-AMP, the cell viability decreased to ~81, 75, and 63% at 100, 200, and 300 μ M H₂O₂, respectively. However, cells pre-treated with Co-

AMP hydrogel showed elevated cell viability (88, 85, and 81%) when exposed to 100, 200, and 300 μM H_2O_2 . The results suggested that the hydrogel with its catalase activity in the cytoplasm degrades the excess of H_2O_2 , thus protecting cells from oxidative stress (**Figure 3A.8a, b**).

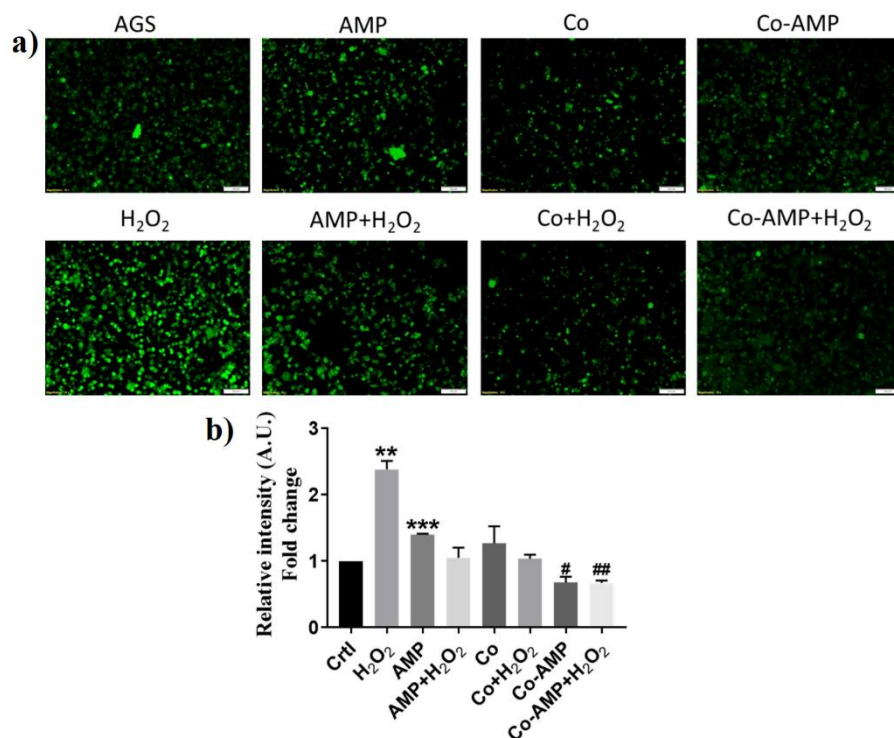


Figure 3A.9 Evaluation of the reactive oxygen species (ROS) production in AGS cells after treatment of compounds and H_2O_2 : a) The fluorescence microscopic image of cells treated with AMP, Co, and Co-AMP for 48 hours, followed by H_2O_2 treatment. In one set only H_2O_2 -treated cells were taken. A set of untreated cells was taken as control, b) Quantification of the generated ROS in cells. Images were captured using a fluorescence microscope. Image analysis and quantification were done using Image J software. The experiment was performed for two biological and two technical replicates, and the results are shown as the mean $\pm$ SD for two independent experiments. Data were statistically analyzed using an unpaired t-test. *p* values were estimated using GraphPad Prism version 7, and *p* values of 0.05, 0.01, and 0.001 were considered statistically significant and represented by */#, **/##, and ***/###, denoting upregulation/downregulation, respectively.

3A.2.7 Co-AMP Hydrogel Scavenges Reactive Oxygen Species from the Cell Cytoplasm

The fluorescence microscope images of H₂O₂-exposed cells show the generation of ROS in the cell cytoplasm, as substantial green fluorescence can be observed as compared to control cells. Cells exposed to only AMP, and Co showed higher green fluorescence, whereas the cells treated with Co-AMP hydrogel showed significantly lower green fluorescence in comparison to AGS cells ($p < 0.05$), suggesting the ability of the hydrogel to prevent ROS generation. Significant green fluorescence was observed from cells treated with H₂O₂ ($p < 0.01$) while pre-treating the cells with Co-AMP hydrogel followed by H₂O₂ resulted in significant decrease in fluorescence ($p < 0.01$) (**Figure 3A.9a, b**). The qualitative microscopic data suggest that the Co-AMP hydrogel could act as a potent antioxidant by regulating ROS-mediated oxidative stress in AGS cells.

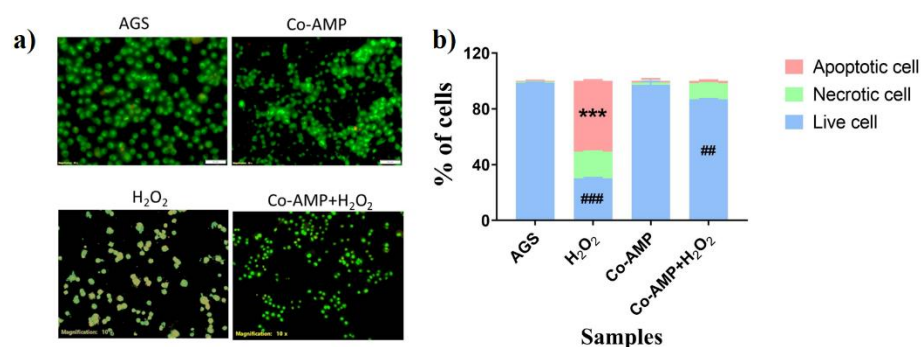


Figure 3A.10 Co-AMP treatment reduces the cancerous properties of AGS cells by fostering programmed cell death: a) A representative image of ethidium bromide and acridine orange (EB/AO) stained AGS cells showing different apoptotic phases after treatment with the compounds followed by exposure to H₂O₂, b) Quantification of the percentage of cells in the different phases of apoptosis using ImageJ software. The experiment was performed for two biological and two technical replicates, and the results are shown as the mean $\pm$ SD for two independent experiments. Data were statistically analyzed using an unpaired *t*-test. *p* values were estimated using GraphPad Prism version 7, and *p* values of 0.05, 0.01, and 0.001 were considered statistically significant and represented by */#, **/##, and ***/###, denoting upregulation/downregulation respectively.

3A.2.8 Co-AMP reduces cell death in AGS cells

Further, apoptosis assays were performed using ethidium bromide and acridine orange stains to analyse the apoptotic cells and quantitatively distinguish them from necrotic cells. Cells were classified into three groups: live, necrotic, and apoptotic cells. The cells treated with H₂O₂ were significantly more prone to apoptosis ($p < 0.001$). However, in the presence of Co-AMP hydrogel more live cells could be observed even after H₂O₂ treatment (Figure 3A.10a, b).

3A.2.9 Expression of antioxidant-associated genes' transcripts

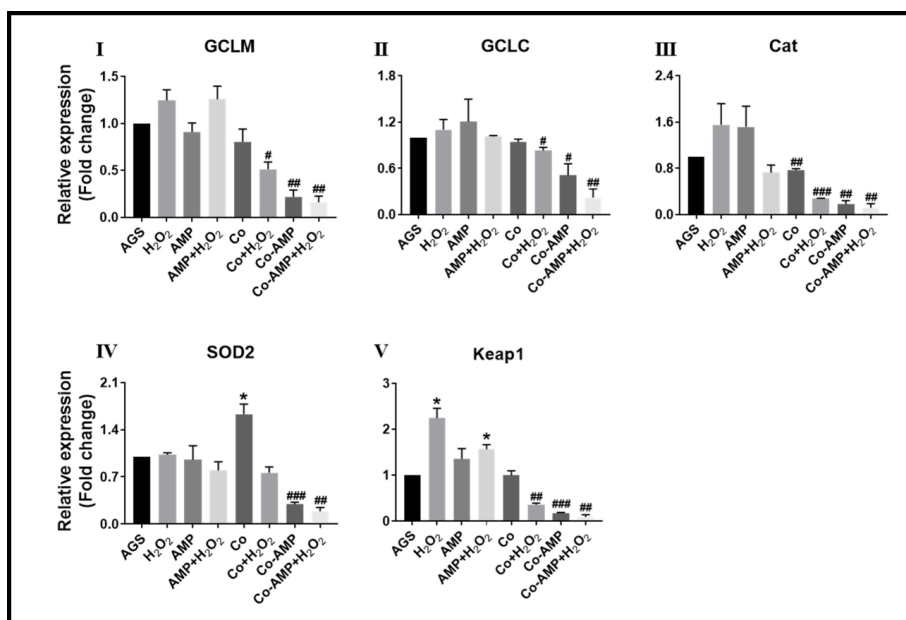


Figure 3A.11 Co-AMP hydrogel treatment modulates the expression profiles of antioxidant genes. Expression pattern of (I) *gclm*, (II) *gclc* (III) catalase (IV) *sod2* (V) *keap1* after pre-treatment with AMP, Co, Co-AMP followed by H₂O₂ exposure. All gene expressions are normalized to the human glyceraldehyde 3-phosphate dehydrogenase gene. The data represent a combination of two biological and two technical replicates. The experiment was performed for two biological and two technical replicates, and the results are shown as the mean $\pm$ SD for two independent experiments. Data were statistically analyzed similar to the other experiments.

qRT-PCR analysis of transcripts of hydrogel treated with AGS cells shows altered expression of antioxidant genes (*gclm*, *gclc*, *sod2*, *cat*, and *keap1*) 48 hrs post-treatment. Whereas elevated expression of all studied antioxidant genes was observed in cells treated with H₂O₂,

we observed a significant decrease in the expression of all studied antioxidant genes in the case of Co-AMP hydrogel and Co-AMP + H₂O₂ treatment ($p < 0.05$, $p < 0.01$). The results suggest that the hydrogel could efficiently regulate the oxidative stress generated in the AGS cells (Figure 3A.11).

3A.3 Conclusion

In conclusion, the Co-AMP hydrogel was highly stable at room temperature and within a wide pH window ranging from 1 to 12. The hydrogel was exploited as an enzyme mimic and displayed pH-dependent catalase and peroxidase-mimicking activity. Whereas in the neutral and basic medium, the hydrogel shows catalase-like activity, this enzymatic behavior was absent under acidic conditions. In an acidic medium, the hydrogel showed efficient peroxidase-like activity that was further utilized for glutathione detection. The multienzyme mimetic behavior of the hydrogel was further demonstrated for phenoxazinone-synthase-like activity. The hydrogel was found to be biocompatible, and its catalase mimic behavior was further utilized to prevent cell apoptosis in the presence of oxidative stress. In vitro studies in the presence of several biomarkers revealed that it could efficiently prevent cell death by degradation of H₂O₂, which was otherwise toxic to the cells. Thus, this fibrillar self-assembled supramolecular cobalt-based metallogel could be applied to various biological and biomedical applications.

3A.4 Experimental Section

3A.4.1 Materials

Adenosine 5'-monophosphate disodium salt (AMP-Na₂), Adenosine 5'-diphosphate disodium salt (ADP-Na₂), Adenosine 5'-diphosphate trisodium salt (ATP-Na₂), Adenine, Adenosine, Cobalt Chloride hexahydrate (CoCl₂·6H₂O), Cobalt nitrate hexahydrate, Cobalt oxychloride hexahydrate, Water HPLC grade, 3,3',5,5'-tetramethylbenzidine (TMB), 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS), MOPS buffer, Europium

Chloride (EuCl_3), Tetracycline hydrochloride, Reduced Glutathione, Rhodamine B, Terephthalic acid, DMSO, Chloroform, Isopropanol, Ethanol were purchased from Sisco Research Laboratory, India (SRL). 30% Hydrogen Peroxide (H_2O_2) was obtained from Avra Synthesis Private Ltd., Dulbecco's minimal essential medium (DMEM), Fetal bovine serum (FBS), Antibiotic solution-penstrep, MTT, PBS, Ethidium bromide: Invitrogen; Acridine orange were purchased from Himedia. H_2DCFDA , Trizol were obtained from Sigma-Aldrich. cDNA was purchased from Takara Bio. All chemicals were used as received without further purification.

3A.4.2 Peroxidase Activity Assay

Peroxidase mimicking activity of Co-AMP was evaluated using TMB as a chromogenic substrate. In this assay, 0.5 mg/mL of Co-AMP gel was added to 3.0 mL of 0.1 M acetate buffer at pH 4, containing 5 mM H_2O_2 and 1 mM TMB. The oxidation of TMB was monitored via an increase in absorbance at 652 nm using a UV-visible spectrophotometer. To determine the kinetic parameters, steady-state kinetics were performed by varying the concentration of TMB from 0.25 mM to 11 mM at a fixed concentration of H_2O_2 (5 mM). Similarly, kinetic analysis was done using H_2O_2 as a substrate, and its concentration was varied (0.1-3 mM), keeping the concentration of TMB fixed at 1 mM.

3A.4.3 Catalase Activity Assay

In this assay, 0.5 mg/mL Co-AMP gel was added to water containing hydrogen peroxide. The released oxygen was measured using an oxygen sensor. Steady-state kinetics experiments were performed by varying the concentration of H_2O_2 from 5 mM to 150 mM, and the various kinetic parameters were calculated. Catalase mimetic activity was also performed using a europium (III)- tetracycline-based assay. Europium Chloride (6.3 mM, 65 μL), tetracycline hydrochloride (2.1 mM, 65 μL), and H_2O_2 (0.3 M, 200 μL) were added to a MOPS buffer solution (10mM, 1670 μL , pH 6.9) and incubated for 10 min. Then, 0.5 mg/mL

Co-AMP gel was added at room temperature. The fluorescence spectrum was recorded with an excitation wavelength of 405 nm.

3A.4.4 Phenoxazinone-synthase assay

The phenoxazinone-synthase-like activity was monitored using o-aminophenol (OAP) as the substrate. In this assay, 1 mg/mL of Co-AMP xerogel was added to 3 mL of methanol containing 0.01 M OAP. The formation of 2-aminophenoxazinone was monitored by an increase in absorbance at 430 nm by a UV-visible spectrophotometer. Kinetic parameters were determined by performing steady-state kinetics in the concentration range of 0.005-0.011 M of OAP.

3A.4.5 Glutathione Detection

For the detection of glutathione, 1.5 mg Co-AMP xerogel was added in 3 mL of acetate buffer (pH 4.0) containing 1 mM TMB and 5 mM H₂O₂. The mixture was incubated for 20 min till the mixture turned blue due to the oxidation of TMB. Then, an aqueous solution of reduced glutathione was added to it, and a change in absorbance was recorded using a UV-Visible spectrophotometer at different time intervals till the blue solution became colorless.

3A.4.6 Terephthalic Acid Assay

For the terephthalic acid (TA) assay, 1 mM TA was added to 3 mL of acetate buffer solution (100 mM, pH 4.0) containing 5 mM H₂O₂, and then the solution was incubated for 15 minutes. The blank data was recorded by exciting the incubated solution at 315 nm. Then, 1.5 mg Co-AMP xerogel was added to the solution, and the emission was recorded at regular time intervals.

3A.4.7 Rhodamine B degradation

For the dye degradation experiment, 0.5 mg/mL Co-AMP xerogel was added to a 3 mL acetate buffer solution (100 mM, pH 4.0) containing Rhodamine B and H₂O₂. The absorbance of the resulting solution was

recorded at 554 nm at different intervals to monitor the degradation of dye in the presence of the catalyst.

3A.4.8 Preparation of Co-AMP-modified carbon cloth electrode

For the Co-AMP modified carbon cloth electrode preparation, 10 mg Co-AMP xerogel was dispersed in distilled water containing 2 mg of activated carbon. 50-100 μL of as-prepared solution of Co-AMP was deposited on a carbon cloth of size $1 \times 2 \text{ cm}^2$. The electrochemical experiments were performed using Co-AMP modified carbon cloth as the working electrode, Ag/AgCl as the reference electrode, and platinum wire as the counter electrode in 100 mM sodium acetate buffer (pH 4.0). Bare carbon cloth was used as a reference.

3A.4.9 Cell Culture Study

AGS (human gastric adenocarcinoma) cells were acquired from the National Center for Cell Sciences (NCCS), Pune, India. The cells were cultured according to earlier literature [19]. Treatment of AMP, CoCl_2 (Co), Co-AMP hydrogel, and H_2O_2 was performed in DMEM media.

3A.4.10 Cell Viability Assay

For the MTT assay, approximately 10,000 cells per well were seeded in 96-well plates. The cells were exposed to AMP, Co, and Co-AMP at different concentrations for 48 h. In a separate experiment, cells were pre-treated with 200.4 μM AMP, 110.64 μM Co, and 156 μM Co-AMP for 48 h, and then H_2O_2 treatment (100, 200, and 300 μM) was given for 2 hr. Afterward, the media was removed from the wells, and 50 μL per well of MTT dye (0.5 mg/mL in PBS) was added and incubated for 3 h at 37 $^\circ\text{C}$. Next, 100 μL of DMSO was added to each well, and absorbance at 570 nm was recorded using a microplate reader (BioTek, Synergy HT).

3A.4.11 H_2DCFDA staining

In a 12-well plate, AGS cells were seeded and treated with the AMP, Co, and Co-AMP hydrogel for 48 hr, followed by 2 hr treatment with H_2O_2 .

Subsequently, cellular ROS production was measured using H₂DCFDA dye as described in earlier literature [20].

3A.4.12 qRT-PCR

After completion of treatment on AGS cells, the pellets were collected, and total RNA was extracted using Tri-reagent (Sigma USA) as per the manufacturer's instructions. cDNA was synthesized using the Takara Prime Script 1st strand cDNA synthesis kit (Takara Japan). qRT-PCR-based gene amplification and a quantification study were carried out as described in the previous study [19,21,22]. Analysis of antioxidant genes (*gclc*, *gclm*, *cat*, *sod2*, and *keap1*) was carried out, using *gapdh* as a reference gene.

3A.4.13 EB-AO staining

The assessment of live, necrotic, and apoptotic cells after exposure to compounds was performed through EB/AO dual staining according to previous literature. Briefly, the cells were stained with EB and AO mixture (100 µg/ml each) for 20 min at 37 °C, followed by PBS wash. Images were taken through a fluorescence microscope (Olympus IX83, Olympus, Japan) at 10X objective magnification. The analysis of the cell stage (apoptotic/ necrotic) was done manually by observing the color characteristics [22].

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Chapter 3B

Cobalt-Adenosine Monophosphate (Co-AMP) Hydrogel as a Non-Zinc and Non-Histidine Carbonic Anhydrase Mimic

3B.1 Introduction

The exponential rise in the levels of carbon dioxide (CO₂) in the atmosphere is mainly driven by humanity's reliance on the utilization of fossil fuels as the primary source of energy [1,2]. Excessive emission of CO₂ contributes to global warming, melting glaciers, and disrupting the climate balance. This results in severe and persistent effects on the ecosystem [3,4]. Hence, there is an urgent need to mitigate CO₂ and the development of alternative energy sources to maintain a green and sustainable environment [5-7]. For the last two decades, researchers have been trying to develop efficient materials to capture CO₂ as well as to convert it into other valuable products such as methanol, ethanol, formic acid, formaldehyde, etc [8-11]. However, nature is proficient in maintaining a balanced ecosystem in its own distinct manner. Carbonic Anhydrases (CAs) are one of the ubiquitous metalloenzymes present in various organisms that catalyze the hydration of CO₂ to bicarbonate and proton with the highest observed rate constant of 10⁶ M⁻¹s⁻¹ to date [12]. CA also plays a vital role in other physiological processes like acid-base balance, fluid secretion, gluconeogenesis, regulating the hemoglobin function, CO₂ transport, etc [13-16]. The natural CA is a kind of hydrolase containing zinc as the metal center, tetrahedrally coordinated to three nitrogen atoms of histidine residues and one oxygen atom from water or hydroxide. It results in the formation of a (Zn-OH) center that binds the CO₂ molecule and converts it to bicarbonate [17,18]. Natural CA enzymes can mitigate CO₂ selectively and effectively, and their unique catalytic ability has garnered significant attention in fields like biofuels, carbon sequestration systems, and calcite production [19]. It

suffers from certain limitations restricting its implementation at the industrial level, such as high cost of production, tedious extraction from organisms, pH and temperature sensitivity to *in vivo* environments, stability, recovery, and recyclability, etc., which encourage the scientific community to explore the CA mimics to circumvent the above limitations [20,21].

Several CA mimics, such as metal-organic frameworks (MOFs), nanomaterials, polymers, etc., have been developed to replicate the metal active center, ligands, and coordination geometry similar to natural CA [22-25]. However, certain features, such as structural rigidity and instability in physiological conditions, compromise their ability to reproduce the cellular environment accurately. In natural enzymes, tertiary proteins play a pivotal role by providing flexibility to the active center and facilitating the access of substrates, along with the transfer of protons [26,27]. In this regard, biomolecular assemblies and soft materials could serve as an ideal supramolecular network to closely emulate the environment akin to natural CA, since their building blocks and non-covalent interactions involved in self-assembly mirror the characteristics of natural protein. Biomolecule-derived hydrogels could offer a biologically relevant platform to replicate the intracellular microenvironment owing to their stability in aqueous medium, structural flexibility, and biocompatibility. The hydrophilic nature of hydrogels also enables them to retain high-water content, resembling the hydrated nature of biological tissues [25,28,29]. Among the various CA mimics developed so far, most of them contain zinc as the active metal center, similar to natural CA [30-37]. Some studies also investigated the effect of native metal substitution in natural carbonic anhydrase utilizing theoretical and simulation methods, and thus creating new avenues for the development of non-zinc CA mimics. Other physiologically relevant metal ions (Co^{2+} , Ni^{2+} , Cu^{2+}) can replace the native metal (Zn^{2+}) in natural CA, which alters their catalytic activity owing to their different coordination geometries [38,39]. Some studies indicated that the metal

complex must maintain a tetrahedral geometry for the hydration reaction of CO₂ to proceed favourably in CAs.

Taking all factors into account, herein, we opted to utilize a cobalt-nucleotide-based hydrogel as a carbonic anhydrase mimic. In this hydrogel, cobalt is coordinated to adenosine monophosphate (AMP) in a tetrahedral fashion. This tetrahedral mode of coordination of metal, along with the nitrogen-rich AMP crosslinked matrix, facilitated the CA mimicking activity of the Co-AMP hydrogel. It exhibited CA-like biocatalytic activity towards both ester hydrolysis and hydration of CO₂ in aqueous medium. The hydrogel also shows stability in a wide pH window and even at elevated temperatures. This thermal and pH stability makes it suitable for industrial conditions. The facile fabrication of this hydrogel using pristine nucleotide with no synthetic steps is also an added perk that increases its appeal. To the best of our knowledge, this is the only CA mimic that does not contain either zinc or histidine moieties as the active center to date.

3B.1 Results and Discussion

The synthesis and characterization of the Co-AMP hydrogel have already been discussed in Chapter 2 in detail. This section focuses on evaluating the esterase and CO₂ hydration activity of the Co-AMP hydrogel. Furthermore, the hydrogel was employed for the biomineralization of metal ions, aimed at facilitating CO₂ sequestration.

3B.1.1 Catalytic Esterase Activity of Co-AMP hydrogel

The CA-mimicking activity of Co-AMP gel was initially evaluated by monitoring ester hydrolysis, which is a well-studied model assay for CA mimics as it is mechanistically analogous to CO₂ hydration [40-42]. During the reaction, p-nitrophenylacetate (pNPA) is converted to p-nitrophenol (pNP). pNPA acts as a colorimetric substrate as it changes its color from colorless to yellow during the reaction (**Figure 3B.1a**). The progress of the reaction can be monitored spectrophotometrically by recording the optical absorbance at 400 nm.

The addition of Co-AMP gel (1 mg/mL) to the solution containing 1 mM pNPA in phosphate buffer (0.01 mM, pH 8) resulted in yellow coloration of the solution. Further, the UV-Visible spectra reveal that the absorbance at 400 nm intensifies with time, which corresponds to the characteristic peak of pNP formation, confirming the intrinsic esterase activity of the Co-AMP xerogel (**Figure 3B.1b**). On the contrary, no color change nor any change in absorbance was observed in the absence of Co-AMP xerogel even after a few days under similar conditions (**Figure 3B.1c**). Further, the kinetic parameters for the esterase activity of gel were calculated using steady-state kinetics, where the concentration of pNPA was varied from 0.2 mM to 2.5 mM. The rate of reaction was plotted against the variable concentrations of pNPA, which follows a typical Michaelis-Menten curve, demonstrating the enzyme-like characteristics of Co-AMP gel. The calculated kinetic parameters, Michaelis constant (K_m) and maximum velocity (V_{max}), were 1.08 mM and 2.51×10^{-4} mM/s, respectively (**Figure 3B.2a**). The obtained kinetic parameters suggested a high affinity of the gel towards CO_2 , which is lower than that of natural carbonic anhydrase.

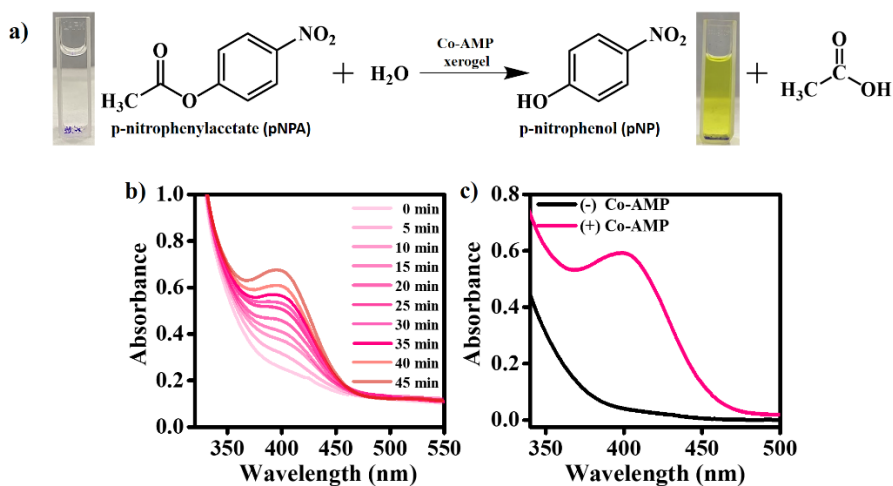


Figure 3B.1 a) Schematic illustration depicting esterase activity of Co-AMP xerogel, b) Time-dependent hydrolysis of pNPA to pNP for 45 minutes, c) UV-visible spectra for pNPA hydrolysis in the presence and absence of Co-AMP xerogel.

The versatility of the catalyst was investigated by exploring a variety of substrates containing different chain lengths, such as 4-nitrophenyl acetate (pNPA), 4-nitrophenyl butyrate (pNPB), 4-

nitrophenyl octanoate (pNPO), and 4-nitrophenyl trifluoroacetate (pNPTFA). The observed reaction rate was in the order ($V_{\text{pNPA}} > V_{\text{pNPB}} > V_{\text{pNPO}}$), suggesting that the rate of reaction decreases proportionally with the increase in chain length of the substrates. Further, the substrate with a bulky electron-withdrawing group (pNPTFA) and a chemically distinct substrate (pNPP) showed almost no catalytic activity, clearly depicting the substrate-specific catalytic activity (**Figure 3B.2b**).

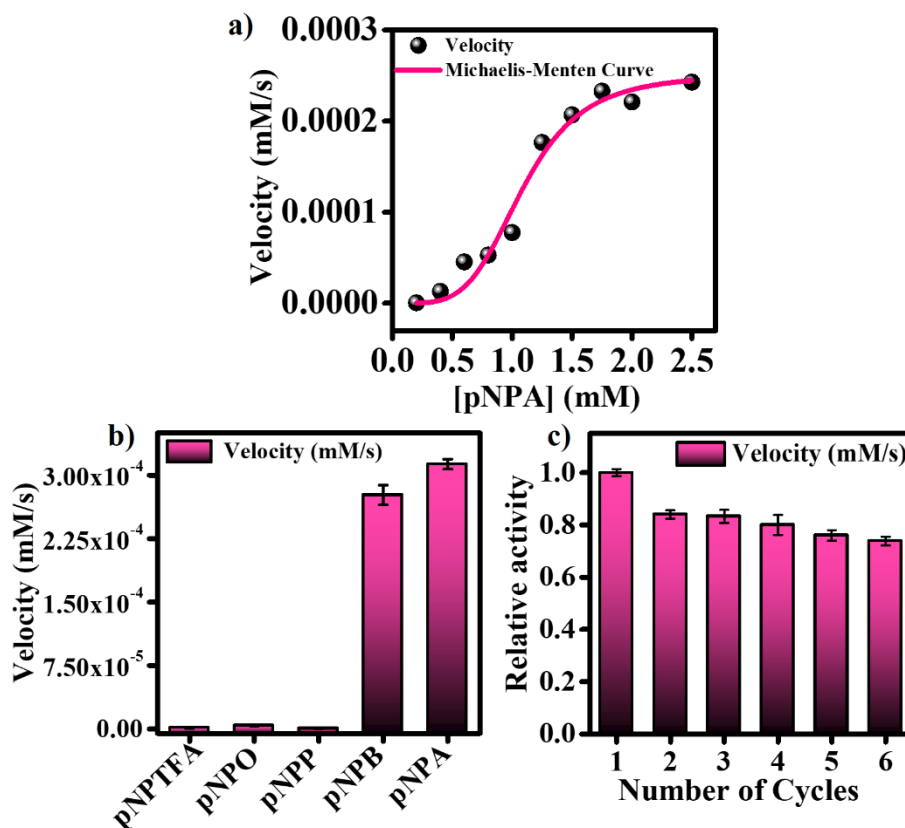


Figure 3B.2 a) Steady state kinetic assay for esterase activity of Co-AMP xerogel with respect to concentration of pNPA, b) Hydrolysis rate of different varieties of nitrophenyl esters, c) Recyclability study for esterase mimicking activity of Co-AMP xerogel.

One of the key features of a catalyst is its reusability. Owing to the heterogeneity of the catalyst, we investigated the recyclability of Co-AMP xerogel by centrifuging it after one cycle. Then it was washed with DI water and methanol, followed by proper drying, and was reused by following the similar procedure for 6 cycles. The catalytic efficiency was retained up to 74 % after 6 cycles (**Figure 3B.2c**). It is well-established that enzymes are highly specific and operate optimally under

a certain range of pH, temperature, and ionic strength. Keeping this in mind, we also explored the effect of temperature and pH on the catalytic activity of Co-AMP xerogel. With an increase in the pH and temperature of the medium, the catalytic activity increased exponentially (**Figure 3B.3a, b**).

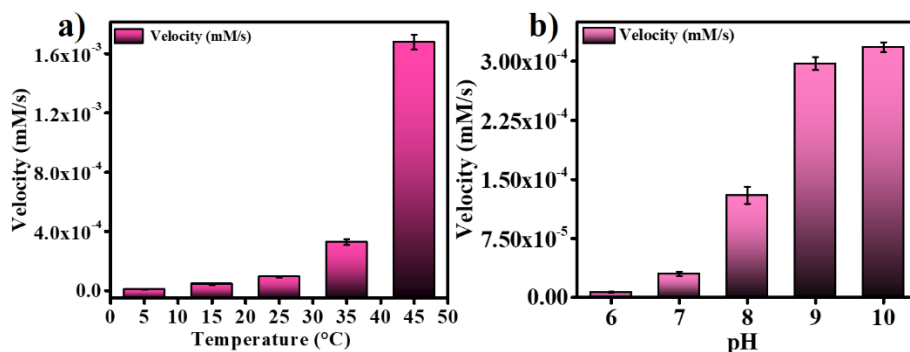


Figure 3B.3 a) Effect of temperature and pH on the CA mimicking activity of the Co-AMP xerogel.

This exponential increase in catalytic activity of Co-AMP xerogel suggests its potential suitability to withstand the harsh industrial conditions, whereas natural enzymes are prone to lose their catalytic activity due to denaturation when exposed to such extreme temperature and pH conditions.

3B.1.2 CO₂ Hydration Activity of Co-AMP Hydrogel

Carbonic anhydrase is a well-known enzyme to catalyze the CO₂ hydration. To demonstrate the CA mimicking activity, we investigated the CO₂ hydration activity of Co-AMP gel. The hydrolysis of CO₂ produces carbonate ions and protons, leading to acidification of the solution, which is reflected by a decrease in pH of the medium. Hence, we measured the change in pH of the medium using a pH meter while purging the CO₂ in the absence and presence of the catalyst. A significant decrease in pH from 8.04 to 5.17 was observed in the presence of Co-AMP gel within 150 seconds, while pH reduced to 7.15 in the absence of the catalyst under similar conditions (**Figure 3B.4a**). This drastic reduction in pH in the presence of gel confirms the CO₂ hydration activity of Co-AMP gel.

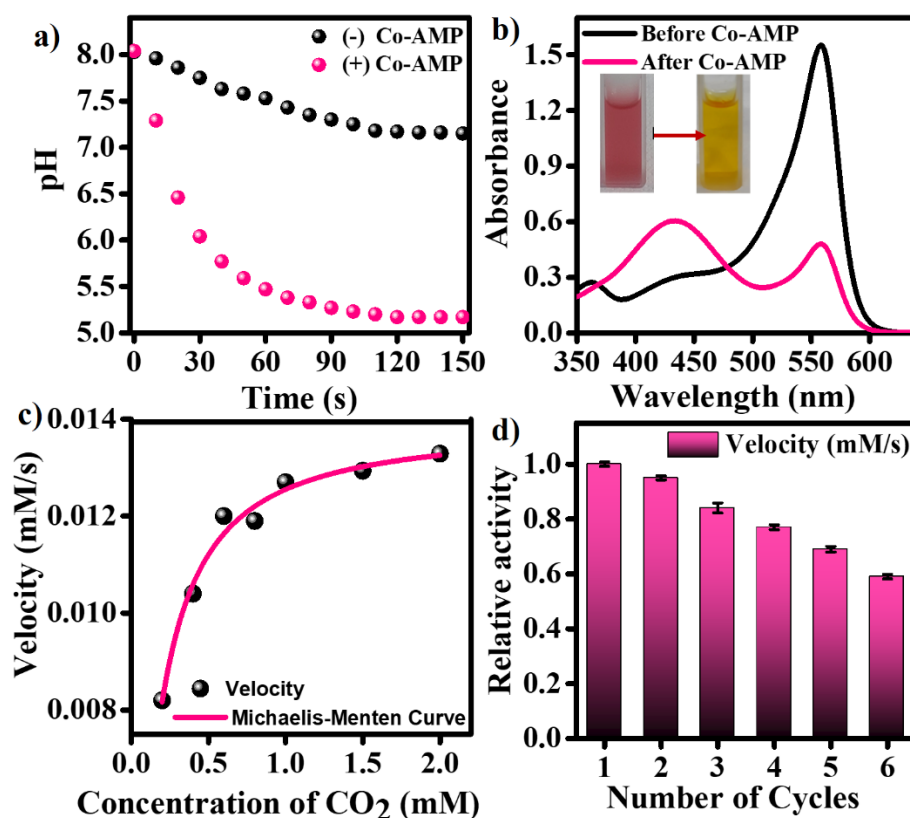


Figure 3B.4 a) Change in pH under CO₂ bubbling conditions in the absence and presence of Co-AMP xerogel, b) UV-visible spectra using phenol red as a pH indicator before and after the addition of Co-AMP xerogel, c) Steady state kinetics upon varying the concentration of CO₂, d) Recyclability of the Co-AMP xerogel up to 6 cycles.

To substantiate the decrease in pH associated with CO₂ hydration, spectrophotometric analysis was conducted using phenol red (PR) as a pH indicator. Initially, 3mM CO₂ was dissolved in phosphate buffer (0.01 mM, pH 8) containing 50 μM PR, and the UV-Visible spectra were recorded before and after the addition of Co-AMP gel. Before the addition of gel, the solution was pink, which is characteristic of an alkaline medium with an intense absorption band at 559 nm and a weak band at 430 nm. Interestingly, after the addition of Co-AMP gel, the solution turned to pale yellow, marked by a diminished intensity at 559 nm and accompanied by an increased intensity at 430 nm, indicating an acidic medium (**Figure 3B.4b**). This observation confirms the CO₂ hydration catalytic activity of Co-AMP gel and thereby induces the pH change from alkaline to acidic. The steady-state kinetics experiments

were performed at a variable concentration of CO₂. The data were well fitted in the Michaelis-Menten Model, and the calculated kinetic parameters such as K_m and V_{max} were 0.14 mM and 1.38×10^{-2} mM/s, respectively (**Figure 3B.4c**). The K_m value for natural CAs vary in the range of 4-18 mM depending on the variants. The K_m value of 0.14 mM and $V_{max} = 1.38 \times 10^{-2}$ mM/s is remarkable as this outperforms all the reported CA mimics to date. The Co-AMP gel shows enhanced affinity towards CO₂ with the fastest kinetics rate as evident from the calculated kinetic parameters, establishing Co-AMP hydrogel as one of the most efficient enzyme-mimicking systems for CO₂ hydration. The catalyst was recycled and reused for 6 cycles with a catalytic activity of 59% (**Figure 3B.4d**).

3B.1.3 Biomineralization of metals via CO₂ sequestration

Carbon mineralization is an emerging field of research that ensures a permanent way to fix CO₂. It involves the reaction of CO₂ with mineral cations to form stable carbonate minerals. It is a prevalent phenomenon in the biosphere in various organisms, such as algae, bacteria, vertebrates, etc., for the formation of stable carbonate mineral structures like skeletons and shells. This is the natural process for mitigating CO₂ levels while having the potential to produce valuable by-products [43,44]. In this context, the CO-AMP gel was utilized to perform the mineralization process of biologically relevant as well as toxic metals while bubbling CO₂ in the system.

Formation of carbonate hydroxyapatite (CO₃Ap)

Biomineralization was performed in phosphate buffer (pH 7.4) to simulate the physiologically relevant conditions. The addition of calcium chloride into CO₂-saturated water under bubbling conditions resulted in the formation of white insoluble precipitate. The precipitate was characterized to confirm its composition. The FESEM images of the calcium-derived mineral revealed sheaf-like morphology, and EDS and elemental mapping confirmed the presence of Ca, C, O, and P (**Figure 3B.5a-g**). It indicated the formation of carbonated hydroxyapatite,

which closely resembles the chemical composition of bone and is used in bone regeneration owing to its better resorption. The formation of carbonated hydroxyapatite was further validated by PXRD analysis, where the pattern exhibits characteristic peaks that match well with the standard JCPDS data. (Figure 3B.5h)

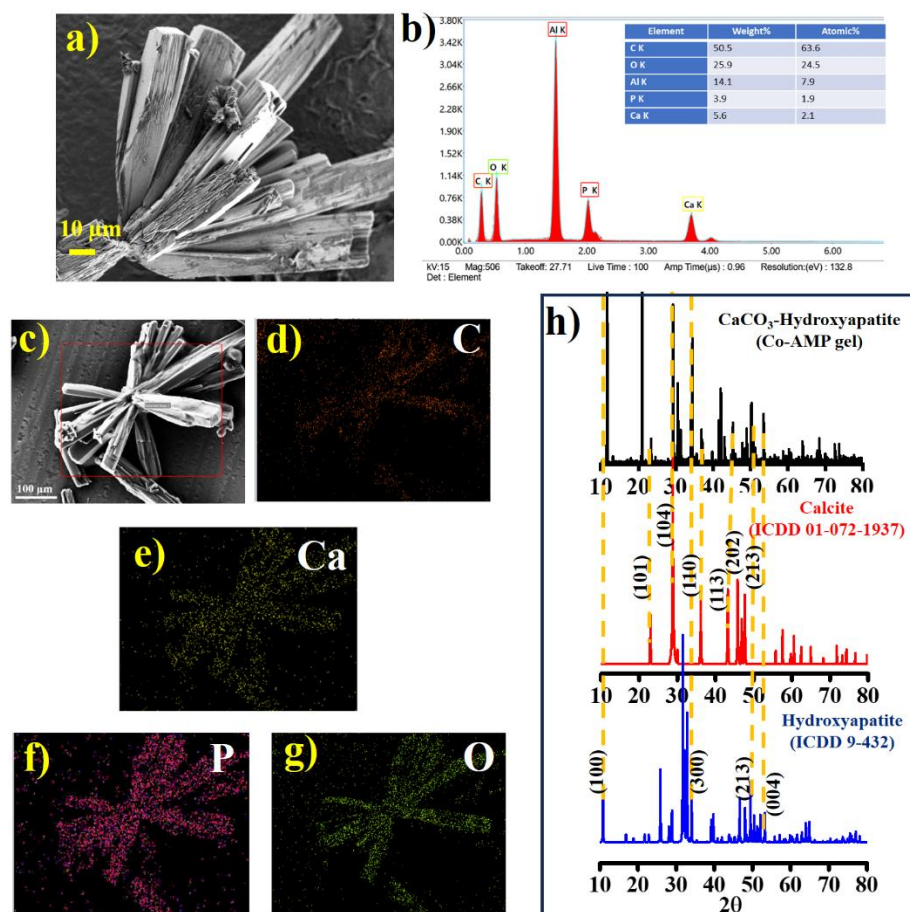


Figure 3B.5 a) FESEM image, b) EDS analysis, c-g) Elemental mapping showing the presence of C, Ca, P, and O, respectively, h) PXRD of the mineralized carbonated hydroxyapatite.

Formation of Barium Carbonate

The barium chloride was used for the biomineralization, and a similar procedure was followed as described in the previous section. The obtained white precipitate was filtered, washed, and dried. It was characterized to confirm the formation of BaCO_3 . The FESEM image showed a rosette-like morphology (Figure 3B.6a). EDS and elemental mapping confirm the presence of Ba, C, and O uniformly distributed throughout the structure (Figure 3B.6b-f). The PXRD pattern matches

well with the reported JCPDS data, confirming the formation of BaCO_3 (Figure 3B.6g).

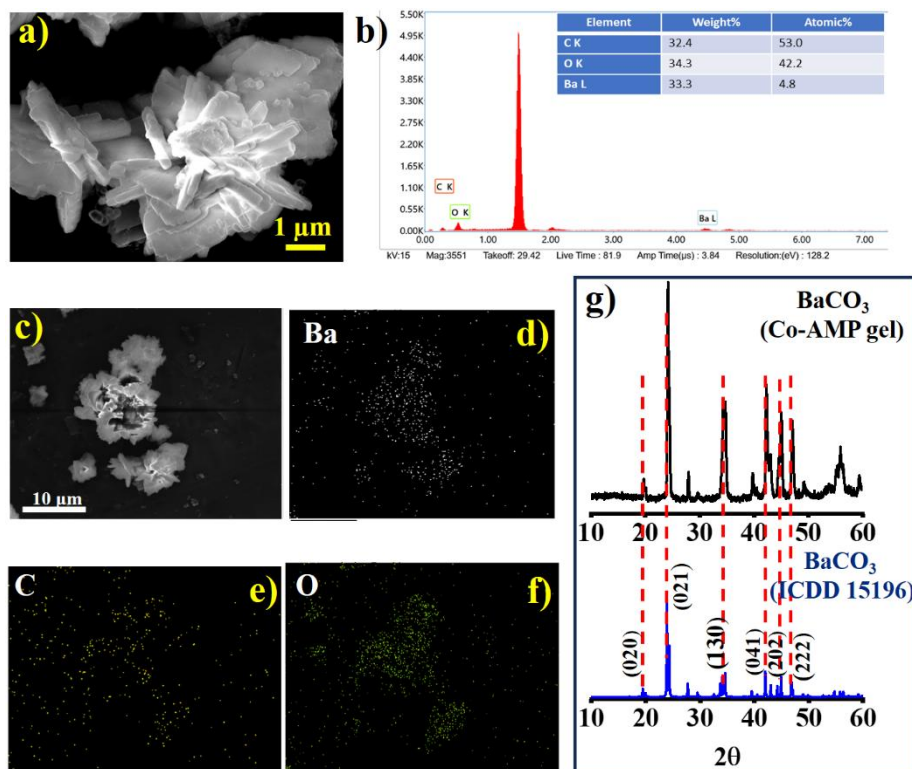


Figure 3B.6 a) FESEM image, b) EDS analysis, c-g) Elemental mapping showing the presence of Ba, C, and O, respectively, h) PXRD of the Barium Carbonate.

Removal of toxic metals using biomineralization

Cd^{2+} and Pb^{2+} are high-profile, toxic heavy metals known for their persistence, bioaccumulation, and non-biodegradability. These can cause severe damage to the living organisms through the food chain [45]. Thus, it is required to prevent their release into the environment. Biomineralization offers a promising strategy for the immobilization of these metal ions from the contaminated water, along with the mitigation of CO_2 . The chloride salts of Cd^{2+} and Pb^{2+} were biomineralized as CdCO_3 and PbCO_3 , respectively. The FESEM image revealed coral-like morphology in the case of Cd^{2+} (Figure 3B.7a). EDS and elemental mapping showed the uniform distribution of Cd, C, and O (Figure 3B.7b-f). The PXRD pattern closely matches the reference JCPDS file, confirming the formation of crystalline CdCO_3 (Figure 3B.7g).

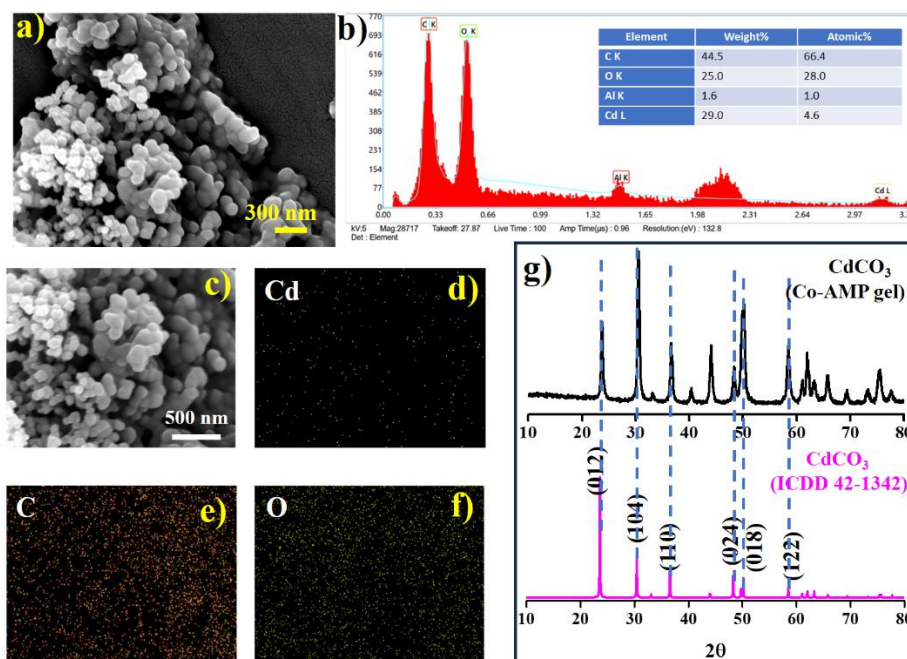


Figure 3B.7 a) FESEM image, b) EDS analysis, c-g) Elemental mapping showing the presence of Cd, C, and O, respectively, h) PXRD of the CdCO₃.

The FESEM analysis demonstrated the presence of spherical architectures in the case of Pb²⁺. The elemental mapping and EDS analysis revealed the presence of Pb, C, and O throughout the structure.

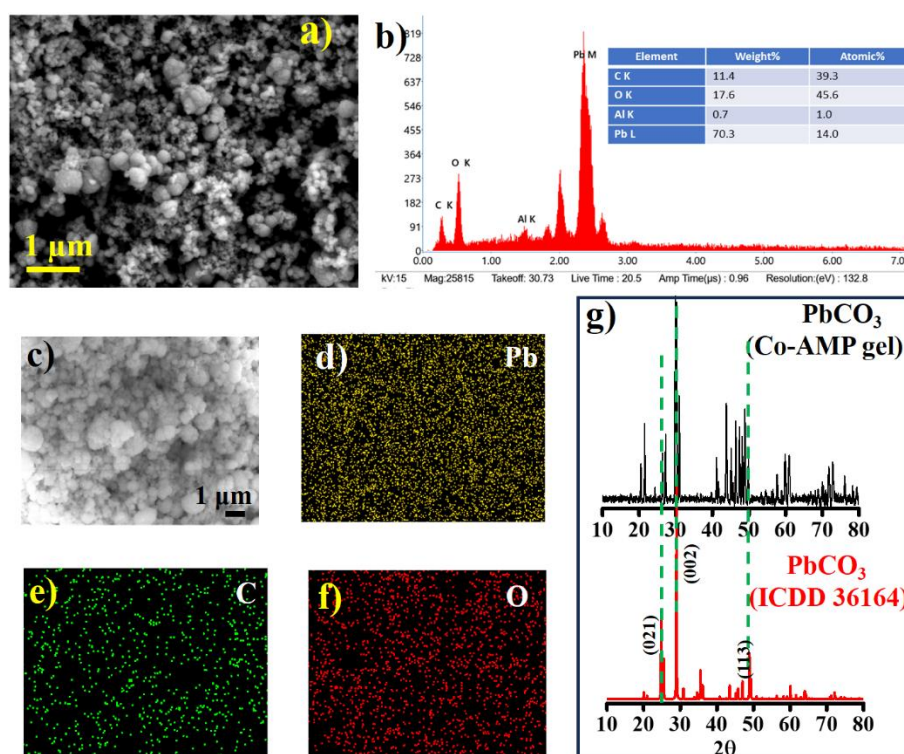


Figure 3B.8 a) FESEM image, b) EDS analysis, c-g) Elemental mapping showing the presence of Pb, C, and O, respectively, h) PXRD of the PbCO₃.

The PXRD pattern confirmed the formation of PbCO_3 with a diffraction pattern matching the standard JCPDS reference file (**Figure 3B.8a-g**).

3B.2 Conclusion

In conclusion, the multifunctionality of the Co-AMP is demonstrated by its CA-mimicking activity. It showed efficient catalytic activity for both esterase and CO_2 hydration activity. The hydrogel showed better substrate affinity than the natural enzyme. This is the first report of a non-zinc and non-histidine CA mimicking system. It can be reused and recycled for several cycles. CO_2 sequestration was performed via biomineralization of biologically relevant metal, like Ca^{2+} . It was further extended for the removal of toxic heavy metal ions such as Cd^{2+} and Pb^{2+} . The facile synthetic strategy, stability, and high efficiency of this hydrogel make it suitable for industrial implications and a significant contribution towards the development of advanced non-native metal-containing CA mimicking catalytic systems.

3B.3 Experimental Section

3B.3.1 Materials

4-nitrophenyl acetate, 4-nitrophenyl butyrate (pNPB), 4-nitrophenyl octanoate (pNPO), and 4-nitrophenyl trifluoroacetate (pNPtFA) were procured from BLD Pharma. Metal chlorides, including calcium (II) chloride dihydrate, cadmium (II) chloride anhydrous, and barium (II) chloride dihydrate, were purchased from SRL Chemicals, India. Lead (II) chloride was purchased from Sigma Aldrich. Phenol Red, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, and sodium hydroxide were purchased from Finar, India. Milli-Q water was used throughout the experiments. All chemicals were used as received without further purification.

3B.3.2 Esterase activity and calculation of kinetic parameters

The esterase activity of the Co-AMP xerogel was investigated for the hydrolysis reaction using 4-nitrophenyl acetate (pNPA) as the

model substrate. The reaction rate was measured by analyzing the formation of 4-nitrophenol (product) using UV-visible spectroscopy. For this, 1 mg/mL of the catalyst was added to phosphate buffer (100 mM, pH 8) containing 1 mM pNPA solution. The hydrolysis was monitored by recording the absorbance at $\lambda = 400$ nm at regular intervals of time after incubation for 2 minutes. The stability of the catalyst was evaluated by monitoring the catalytic activity at different temperatures and pH. Steady-state kinetic parameters were calculated by varying the concentration of pNPA. The recyclability of the catalyst was assessed by centrifuging the catalyst after each cycle. The supernatant was separated, and the catalyst was washed with deionized water and then reused for the next cycle.

3B.3.3 CO₂ hydration activity assay

The CO₂ hydration activity of the Co-AMP xerogel was systematically evaluated. To prepare carbonated water, pure CO₂ gas was bubbled at a flow rate of 200 mL/min into 200 mL of deionized water in an ice bath for 2 hours using a CO₂ flowmeter. Based on Henry's law, the maximum solubility of CO₂ in water at 273.15 K and 1 atm is 3.48 g/L, corresponding to a dissolved CO₂ concentration of 77 mM. For catalytic activity measurements, phenol red (50 μ M) was employed as a pH indicator, and the Co-AMP catalyst was dissolved in phosphate buffer (100 mM, pH 8). The hydration of CO₂ was carried out using various concentrations and dilutions of carbonated water, using Co-AMP as a catalyst. The progress of the reaction was monitored through a colorimetric method by monitoring the change in absorbance at 560 nm over time. Initial reaction rates (V_0) were extracted from the kinetic data corresponding to different CO₂ concentrations. These V_0 values were plotted as a function of substrate concentration, and the Michaelis–Menten parameters, $V_{\max}$ and K_m , were determined accordingly.

Additionally, continuous pH was monitored for the buffer containing Co-AMP xerogel (1 mg/mL) under a constant CO₂ flow rate

of 50 mL/min. The change in pH over 150 seconds was recorded using a LABMAN LMPH10H pH meter. Control experiments were carried out under identical conditions using individual catalyst components and in the absence of any catalyst, to validate the catalytic role of Co-AMP in CO₂ hydration.

3B.3.4 Biomineralization of the metal carbonates via CO₂ sequestration

The biomineralization of various metal carbonates—including calcium carbonate, barium carbonate, cadmium carbonate, and lead carbonate—was catalyzed using the CA-mimicking Co-AMP xerogel. The mineralization process was carried out by gently bubbling CO₂ gas (50 cm³/min) into a phosphate buffer solution (100 mM, pH 7.4) containing metal chlorides (10 mM) and Co-AMP xerogel (1 mg/mL) for 60 minutes. Following CO₂ purging, the reaction mixture was allowed to stand undisturbed for 12 hours to facilitate the precipitation of metal carbonates. The resulting precipitates were thoroughly washed with deionized water to remove any residual catalyst and unreacted metal salts. After drying, the mineralized products were characterized using scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), and powder X-ray diffraction (PXRD).

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Chapter 3C

A Tetrahedral Coordinated Cobalt Adenosine Monophosphate Hydrogel as a Bifunctional Electrocatalyst for Overall Water Splitting

3C.1 Introduction

The global shift toward green and sustainable development has intensified efforts to advance clean energy technologies that minimize environmental impact [1]. Rapid industrialization and rising energy demands have accelerated the search for eco-friendly alternatives to fossil fuels [2-4]. Among these, electrochemical water splitting is a promising route for sustainable hydrogen (H_2) and oxygen (O_2) production. The process comprises two half-reactions: the hydrogen evolution reaction (HER) and the oxygen evolution reaction (OER) [5]. Of the two, the OER is typically rate-limiting due to its sluggish proton-coupled electron transfer kinetics and the high energy barrier associated with O–O bond formation [6,7].

Noble metal-based catalysts such as RuO_2 , IrO_2 , Pt, and Pd currently set the benchmark for OER and HER. However, their high cost, scarcity, and limited scalability hinder their industrial application. This has prompted intensive efforts to develop low-cost, earth-abundant catalysts with high electrocatalytic activity and long-term durability [8]. Transition metal-based materials—such as oxides, hydroxides, oxyhydroxides, phosphides, MOFs, and COFs—have shown promise in this regard [9-17]. Yet, their synthesis often requires strict control over morphology, crystallinity, and porosity to ensure consistent performance. Therefore, the development of noble metal-free catalysts via simple, scalable, and cost-effective synthetic routes remains

essential for advancing practical and sustainable water splitting technologies [18,19].

Key structural features like porosity, high surface area, and superior electrical conductivity are essential for the development of efficient electrocatalysts, as they facilitate efficient ion and electron transport and accelerated mass transfer kinetics [20,21]. In this context, hydrogels represent a promising class of material fulfilling all these stringent structural and functional requirements; however, their potential in electrocatalysis remains largely unexplored. Their highly hydrated, swellable polymeric network ensures the electrolyte confinement, facilitating the rapid access of reactant molecules to the catalytically active site [22]. Moreover, the self-healing behaviour of hydrogels preserves catalyst integrity, preventing structural and performance degradation even under prolonged harsh operational conditions [23-27]. These synergistic properties establish nucleotide-based hydrogels as integrated catalytic platforms, unifying electrolyte management, ion transport, and durability within a single matrix.

Cobalt-based catalysts are effective for OER but show limited HER activity. This limitation can be addressed through bimetallic systems that exploit synergistic interactions between different metal centers to enhance the overall catalytic efficacy [28-30]. The phosphate group, which constitutes the backbone of nucleic acid chemistry and is known for its biochemical relevance, enhances water-splitting efficiency by strongly binding key intermediates like protons and hydroxyls [31,32]. Additionally, a tetrahedral coordination environment offers accessible active sites and structural flexibility, facilitating faster reaction kinetics [33-35]. Therefore, the strategic design of coordination polymers using appropriate organic ligands is vital for developing efficient and robust electrocatalysts.

Here, we utilized the Co-AMP hydrogel as a bifunctional electrocatalyst for overall water splitting in 1 M KOH. The tetrahedral arrangement around the cobalt atom, the presence of the phosphate

group as a ligand, and the exceptional stability of this hydrogel collectively render it an ideal candidate or choice for electrochemical water splitting. This structural synergy or integrated design of Co-AMP hydrogel resulted in superior electrocatalytic activity. The thixotropic, self-healing, and swelling behaviour of the hydrogel collectively contribute to the long-term stability of the Co-AMP hydrogel during the repeated redox cycles. Interestingly, the 1:2 Co-AMP hydrogel showed a record low voltage of 1.56 V and 1.42 V at 25 °C and 70 °C, in 1 M KOH, respectively, for overall water splitting. This drastic decrease in cell voltage at high temperature is a distinctive phenomenon and can be attributed to the temperature-assisted formation of the hydrogel. The facile, straightforward synthetic methodology and exceptional stability of this nucleotide-based hydrogel promote its applicability in harsh industrial conditions. This hydrogel exemplifies the optimal convergence of critical parameters and offers a guiding framework for the rational design of efficient and advanced bifunctional catalysts.

3C.2 Results and Discussion

To investigate the electrocatalytic performance of the Co-AMP hydrogel, four sets of catalysts were prepared by systematically varying the concentrations and molar ratios of the metal precursor (Co^{2+}) and ligand (AMP). Two catalysts, CAH1 and CAH2, were synthesized at a 1:1 ratio of Co: AMP at concentrations of 50 mM and 100 mM, respectively. Another catalyst, CAH3, was fabricated at of 1:2 Co: AMP ratio to assess the effect of ligand on the electrocatalytic activity. Lastly, to investigate the effect of gelation, another catalyst, CAH4, was prepared by physically mixing the aqueous solutions of cobalt and AMP without thermal treatment. The resulting solution was incubated for 7 days at room temperature, followed by freeze-drying to obtain a blue powder. Comprehensive spectroscopic and microscopic analyses indicate that the structural and morphological features of the 1:2 Co: AMP hydrogel are analogous to the 1:1 hydrogel, as elaborated in **Chapter 2**.

Rheological investigations revealed a pronounced influence of fabrication temperature on the mechanical strength and strain-bearing capacity of the Co-AMP hydrogel.

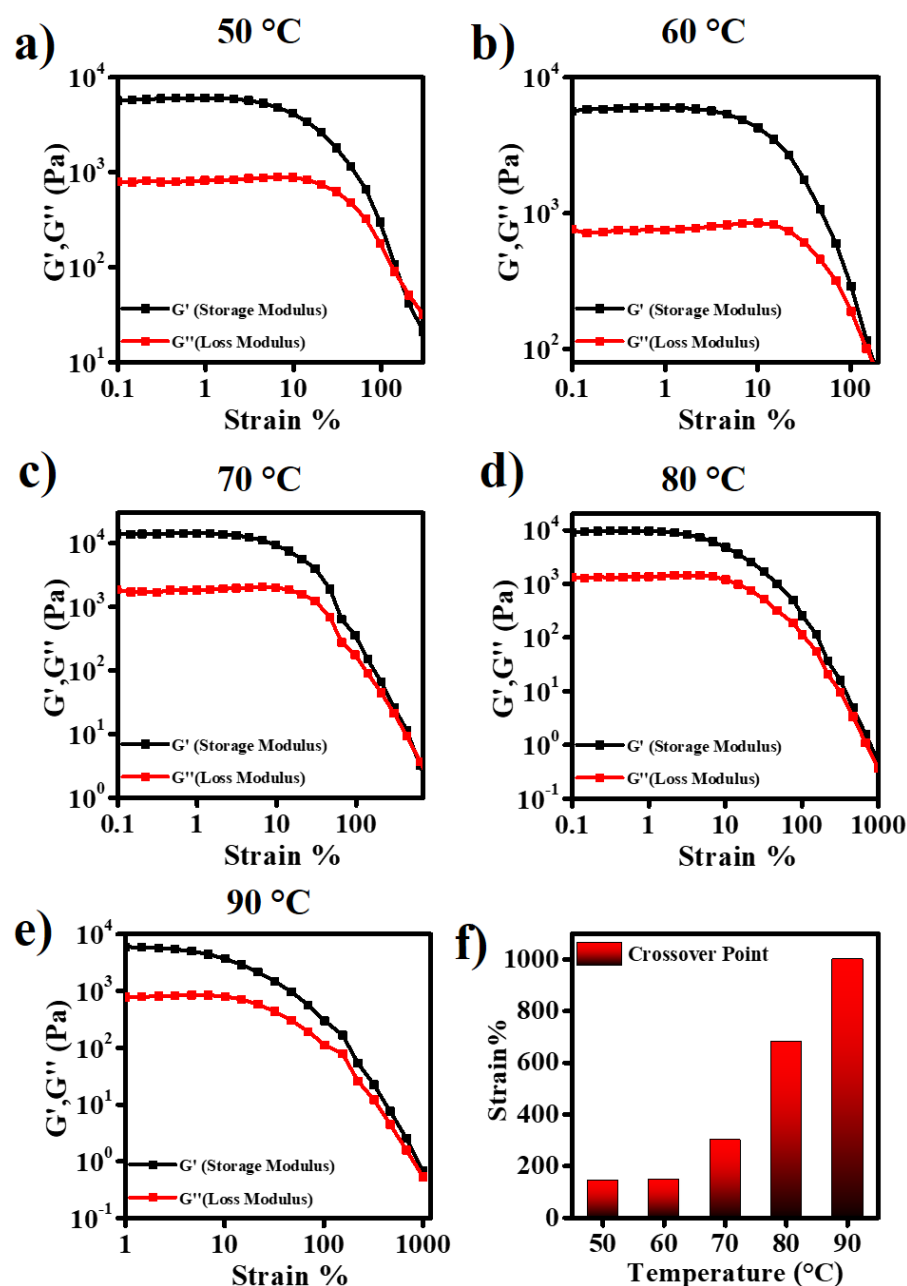


Figure 3C.1 a-f) Amplitude sweep studies of CAH3 fabricated at different temperatures.

Amplitude sweep measurements demonstrated a significant shift in the crossover point—from 142% for the hydrogel formed at 50 °C to 1000% for the one prepared at 90 °C, indicating a substantial enhancement in mechanical robustness with increasing temperature (**Figure 3C.1a-f**).

3C.2.2 Electrocatalytic HER performance of Hydrogels

To assess the electrocatalytic activity of all CAH samples, we thoroughly investigated the HER, OER, and overall water-splitting performances in alkaline electrolyte (1.0 M KOH) with all the potentials calibrated to the reversible hydrogen electrode (RHE).

The iR-corrected (optimized, 85%) cathodic polarization curves for HER of all the studied catalysts, along with benchmark catalyst Pt/C, are shown in **Figure 3C.2a**. CAH3 only demanded 160 mV to produce a current density of 10 mA cm^{-2} against 45 mV for Pt/C, and to our surprise it was found out to be the lowest value of overpotential compared to other as-synthesized catalysts (**Table 3C.1**). The η_{10} values for CAH1, CAH2, and CAH4 were 178 mV, 171 mV, and 167 mV, respectively. Moreover, CAH3 required 275 mV, 400 mV, and 470 mV to achieve current densities of 100 mA cm^{-2} , 500 mA cm^{-2} , and 1000 mA cm^{-2} , respectively. These η values are considerably lower than those required to achieve a commercial-level current density as compared to the other three electrocatalysts, as well as earlier reports based on hydrogel materials. In an alkaline electrolyte, CAH3 offered an insignificant water dissociation energy barrier, which supports the drop in overpotential. Furthermore, the η_{10} for the bare Nickel foam (NF) substrate was 281 mV and reached the maximum current density of 214 mA cm^{-2} (η_{800}) signifying its negligible contribution toward HER. Interestingly, CAH4, the precipitate formed by simple mixing of CoCl_2 and AMP at room temperature, also showed remarkable electrocatalytic activity for HER with an overpotential (η_{10}) of 167 mV. These results highlight the potential of this nucleotide-based self-assembled material as a promising advanced electrocatalyst with minimal and facile synthetic requirements.

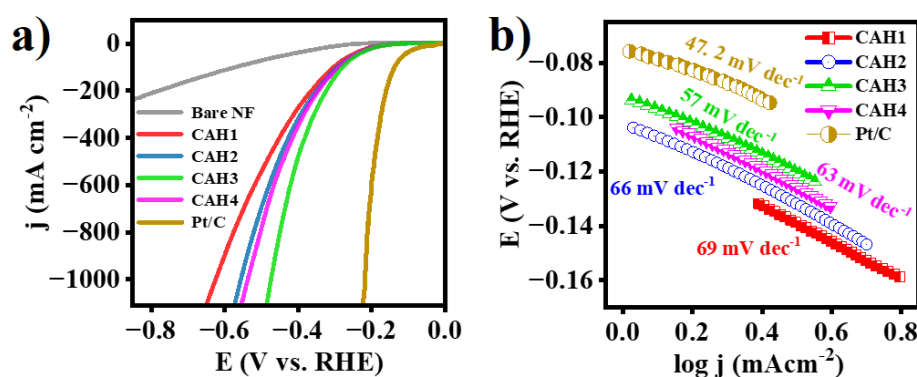


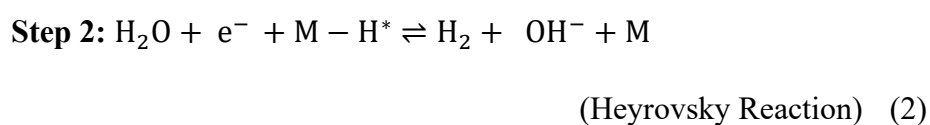
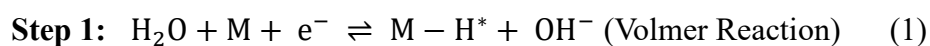
Figure 3C.2 a) Cathodic sweep voltammetry curves for the HER and (b) Corresponding Tafel slope plots for all the samples.

During water electrolysis, catalytic current density (j) rises exponentially with overpotential (η), with variations in the rate of increase for different rate-determining steps (RDS). To analyze the polarization data and dominance of specific HER kinetics, the Tafel slope (b) values, which are related inversely to the reaction rate, were determined for all the catalysts from the linear portion of the steady-state curve as $\log j$ vs η and presented in **Figure 3C.2b**. The Tafel slope of 57 mV dec⁻¹ for CAH3 is smaller among all the samples and relatively close to Pt/C (47.2 mV dec⁻¹), revealing its faster catalytic kinetics among all the compositions (**Table 3C.1**).

S. No.	Catalysts Code	HER		OER	
		Overpotential at 10 mA cm ⁻² (mV)	Tafel slope (mV dec ⁻¹)	Overpotential at 100 mA cm ⁻² (mV)	Tafel slope (mV dec ⁻¹)
1	CAH1	178	69	306	90
2	CAH2	171	66	294	84
3	CAH3	160	57	271	76
4	CAH4	167	63	284	82
5	Pt/C	45	47.2	-	-
6	IrO ₂	-	-	347	104.2
7	Bare NF	281	-	451	-

Table 3C.1 Summary of HER and OER overpotential (mV) and Tafel slope (mV dec⁻¹) of the studied catalysts.

In alkaline electrolytes, the HER involves two reaction pathways (Equations 1-3). The first step is the Volmer step ($b = 120 \text{ mV dec}^{-1}$) which involves the generation of an adsorbed hydrogen intermediate (H_{ads}), by transfer of electron to a proton on active site of an electrocatalyst and the second step involves the formation of H_2 either via electrochemical desorption (Heyrovsky mechanism, $b = 40 \text{ mV dec}^{-1}$) or recombination of two nearby H_{ads} (Tafel mechanism, $b = 30 \text{ mV dec}^{-1}$) [36-39].



The lowest Tafel slope (57 mV dec^{-1}) of CAH3 reveals that the H_2 evolved via the Volmer-Heyrovsky mechanism as a dominant route, and the second electron transfer (electrochemical hydrogen desorption) serves as the rate-determining step (RDS) [40-43]. The low concentration of H_3O^+ ions in an alkaline electrolyte causes H_2O to become the primary HER reactant through additional H-O-H bond dissociation before H^* adsorption, and the low Tafel slope value indicates that there is a high coverage of H^* adsorption over the catalyst surface, which facilitates the release of molecular hydrogen. The high coverage of adsorbed hydrogen species in CAH3 can be attributed to the presence of the phosphate group of AMP and the synergistic coupling of the Co^{2+} and the coordinated ligand, which balances the adsorption and desorption of hydrogen, and offers an ideal free energy of hydrogen adsorption (ΔG_H) for faster water reduction kinetics [44,45].

Electrochemical impedance spectroscopy (EIS) was conducted to compare the electron charge transfer characteristics and investigate the intrinsic resistance of the electrode material and the interfacial resistance between the electrode and electrolyte. The Nyquist plots for all the controls in the frequency range of 0.1 Hz–100 kHz were obtained

by fitting raw data to an appropriate Randles Circuit (**Figure 3C.3a**). **Figure 3C.3b-e** compares the fitted individual plots with equivalent circuits, and **Table 3C.2** provides the fitting parameters in high-frequency regions, where R_{ct} is the charge-transfer resistance, R_b is the bulk resistance, and C is the double-layer capacitance.

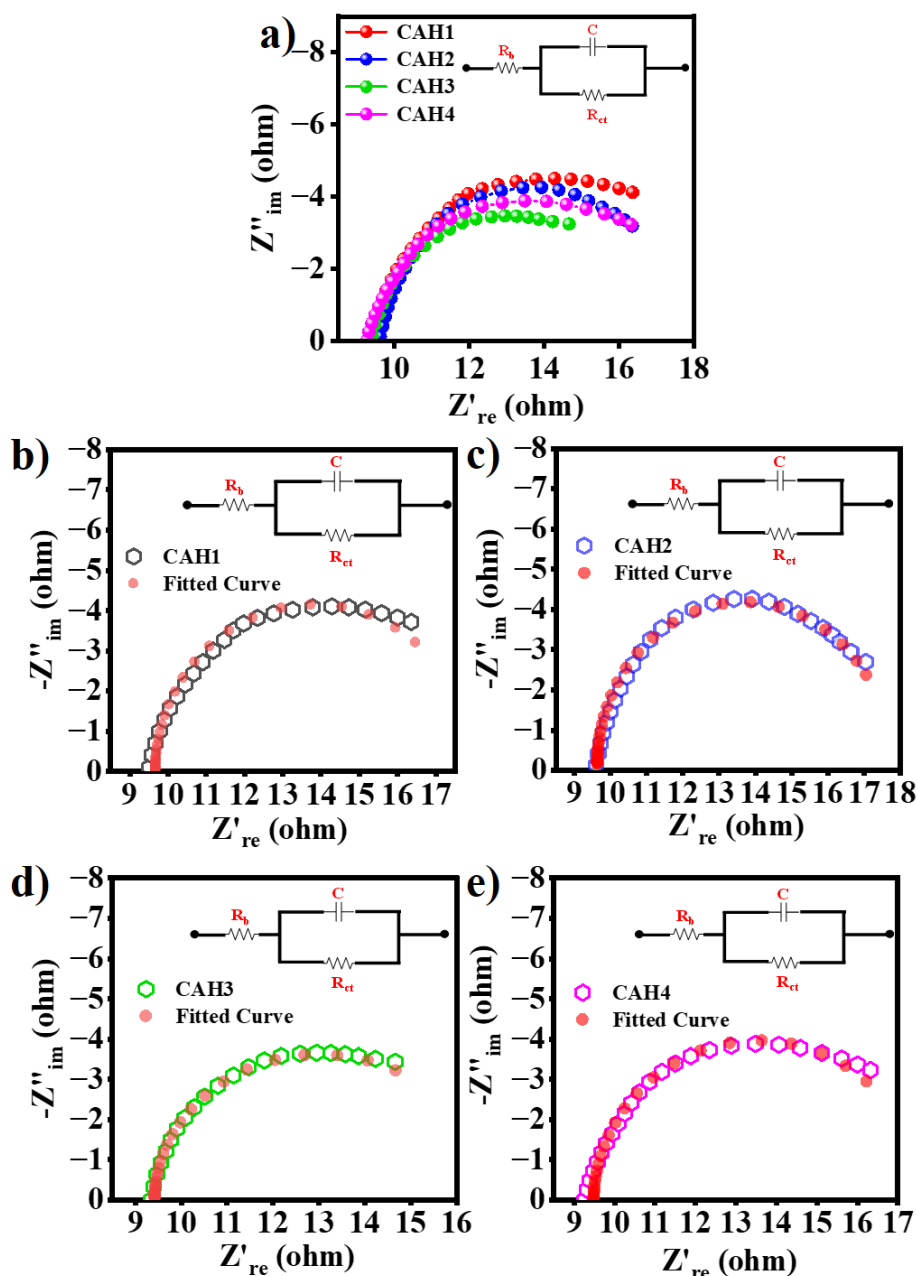


Figure 3C.3 Fitted Nyquist plots along with circuit diagram in case of HER in alkaline electrolyte at an applied potential of -0.18 V vs RHE for: a) all the catalysts, b) CAH1, c) CAH2, d) CAH3, e) CAH4.

S. No.	Catalyst code	R_{ct} (Ω)	R_b (Ω)	C_1 (F)	i_{ex} (mA cm^{-2})
1	CAH1	9.24	9.42	3.83×10^{-5}	15.43
2	CAH2	8.13	9.68	4.05×10^{-5}	17.54
3	CAH3	6.69	9.39	3.54×10^{-5}	21.32
4	CAH4	7.66	9.15	4.37×10^{-5}	18.62

Table 3C.2 Summary of charge transfer resistance (R_{ct}) and the calculated exchange current density (i_{ex}) of the studied catalysts and the EIS equivalent circuit diagram parameters for HER.

The diameter and shape of the semicircle offer detailed information about the ion mobility and charge transfer resistance. It can be observed that the semi-circle diameter of the CAH3 composite material is smaller compared to other hydrogel samples, indicating its superior electron transfer from the electrolyte to the electrode surface for a rapid HER process. The R_{ct} value observed for CAH3 was 6.69 Ω , which was smaller than those observed for CAH1 (9.24 Ω), CAH2 (8.13 Ω), and CAH4 (7.66 Ω). These results support the lower η and b values for CAH3, demonstrating the superiority of this material. The enhanced catalytic activity was ascribed to the modification of the electronic structure due to its delocalization characteristics. Since the ligand ratio is twice in CAH3 as compared to CAH1 and CAH2, there is more room for the delocalization of electrons, which indicates that the electrons do not belong to a specific position but are likely to exist anywhere in the supramolecular network, which improves the charge transfer properties. The role of gelation is also highlighted by the superior activity of CAH3 over CAH4 since the ligand ratio is similar in both cases. The high electrode conductivity and prominent electron migration ability between the two interfaces are justified for CAH3 as it carries the highest exchange current density (i_{ex}) of 21.32 mA cm^{-2} among all variations (Figure 3C.4a, Table 3C.2).

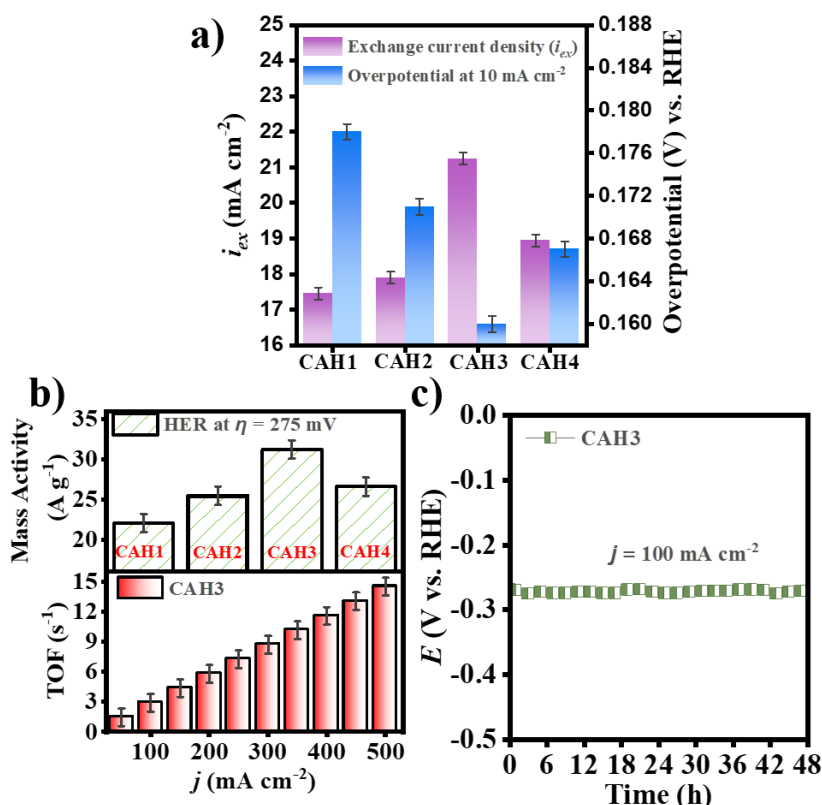


Figure 3C.4 a) Comparison of the exchange current density and HER overpotential at 10 mA cm^{-2} , b) Mass activity (upper) and TOF (lower) of all the samples at the given η , c) Chronopotentiometry curve in the cathodic region.

In addition, the mass activity of all the catalysts was evaluated by normalizing the LSV polarization curves to the catalyst mass loading. The CAH3 catalyst reached the highest mass activity of 31.25 A/g at $\eta = 275 \text{ mV}$ for HER, shown in **Figure 3C.4b (upper panel)**. Based on these data, it was confirmed that for an identical amount of mass loading, CAH3 demonstrated superior activity for the electrolysis. The intrinsic activity of these active sites is highlighted in terms of turnover frequency (TOF) in **Figure 3C.4b (lower panel)**. The turnover frequency of CAH3 was 2.89 s^{-1} for hydrogen evolution at $\eta_{100} = 275 \text{ mV}$. Moreover, the CAH3 electrode demonstrates excellent stability, maintaining consistent activity over 60 hours of chronopotentiometric testing at a high current density of 100 mA cm^{-2} without noticeable degradation (**Figure 3C.4c**).

3C.2.2 Electrocatalytic OER performance of Hydrogels

The bottleneck to water-based electrolysis is the oxygen evolution reaction, as its sluggish kinetics arises from the involvement of its multi-electrons and multi-intermediates during the catalytic process. As shown in anodic sweep voltammetry polarization curves (**Figure 3C.5a**), CAH3 offered an exceptional OER activity. It can afford a current density of 20 mA cm⁻², 100 mA cm⁻², and 500 mA cm⁻² at a consumption of only 215 mV, 271 mV, and 350 mV of overpotential, respectively, which are lower than those of the other CAH samples (**Table 3C.1**). These values are not only significantly lower than those of the bare NF ($\eta_{100} = 451$ mV) but also outperform commercial IrO₂ ($\eta_{100} = 347$ mV). Notably, the CAH3 electrode exhibited the lowest Tafel slope of 76 mV/dec compared to CAH1, CAH2, and CAH4, which is better than IrO₂ (**Figure 3C.5b**).

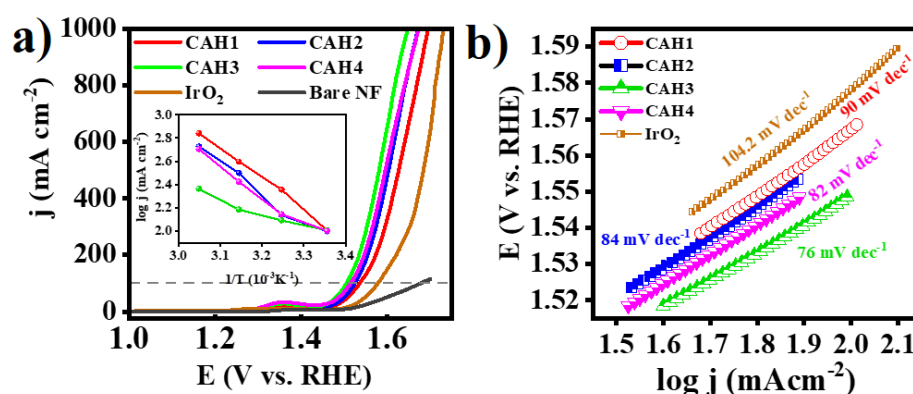


Figure 3C.5 a) Anodic sweep voltammetry curves for the OER (Inset: Arrhenius plots of $\log j$ vs the inverse of temperature.), b) Corresponding Tafel slope plots for all the samples.

In alkaline electrolytes, there could be two possibilities for RDS, with a generally bearing Tafel slope of 120 mV/dec and 40 mV/dec for steps I and II, respectively [46]. The intermediary Tafel slope value suggests that the reaction follows either Step I [$M^*OH + OH^- \rightarrow M^*O + H_2O + e^-$] or Step II [$M^*OOH + OH^- \rightarrow M^* + O_2 + H_2O + e^-$] as the rate-determining step. The higher ligand ratio in CAH3 with increasing

density of the polar phosphate groups can accelerate the rate of OER kinetics by taking part in the chemisorption process.

The Nyquist plots (**Figure 3C.6a**) validated these observations; compared to the other controls, CAH3 possessed the lowest value of R_1 and R_2 , defining the most favorable charge transfer kinetics. Fitted data along with the equivalent circuit, including solution resistance (R_b), charge transfer resistance (R_1), and adsorption resistance (R_2) of all the variations are outlined in **Figure 3C.6b-e & Table 3C.3** [33]. Charge-transfer kinetics also depend upon the double-layer capacitance (C_{dl}) of a particular catalyst, which arises from the charge separation at the electrode-electrolyte interface. The double-layer capacitance (C_{dl}) was determined from CV measurements by varying the scan rates (40 – 80 mV s^{-1}) in a non-Faradaic region. It is directly proportional to the electrochemical active surface area (ECSA) (**Figure 3C.7a-d**). As expected, CAH3 offered the highest C_{dl} of 15.13 mF cm^{-2} and the largest ECSA of 34.05 cm^2 among all the controls, consequently facilitating efficient mass transfer rate (**Figure 3C.7e (left axis), Table 3C.4**). ECSA is the electrode portion that actively participates in the electrochemical reactions, often exceeding the geometric surface area owing to porosity, roughness, and a high surface area to volume ratio of the material. It has the highest roughness factor of 378.33 (**Table 3C.4**). Thus, increasing the ligand concentration in CAH3 furnishes a large active surface site for the diffusion of ions into the electrode material.

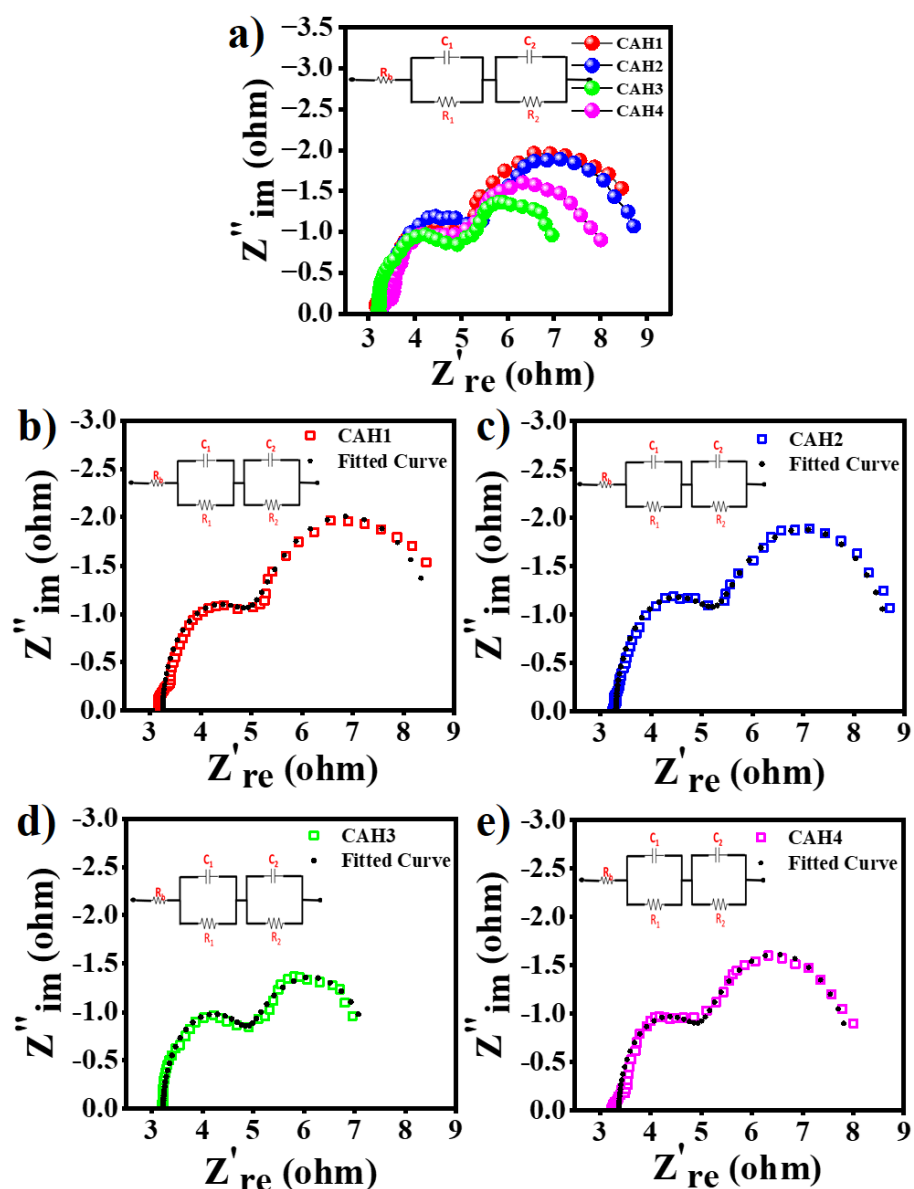


Figure 3C.6 Fitted Nyquist plots along with circuit diagram in case of OER in alkaline electrolyte at an applied potential of 1.53 V vs RHE for: a) all the catalysts, b) CAH1, c) CAH2, d) CAH3, e) CAH4.

S. No.	Catalyst Code	R_b (Ω)	R_1 (Ω)	C_1 (F)	R_2 (Ω)	C_2 (F)	i_{ex} (mA cm^{-2})
1	CAH1	3.23	2.68	0.01691	4.38	0.1676	10.1
2	CAH2	3.22	2.36	0.01338	4.17	0.1212	10.9
3	CAH3	3.23	1.832	0.01131	2.747	0.1407	15.6
4	CAH4	3.22	1.984	0.01452	3.574	0.1741	12.83

Table 3C.3 Summary of charge transfer resistance (R_{ct}) and the calculated exchange current density (i_{ex}) of the studied catalysts and the EIS equivalent circuit diagram parameters for OER.

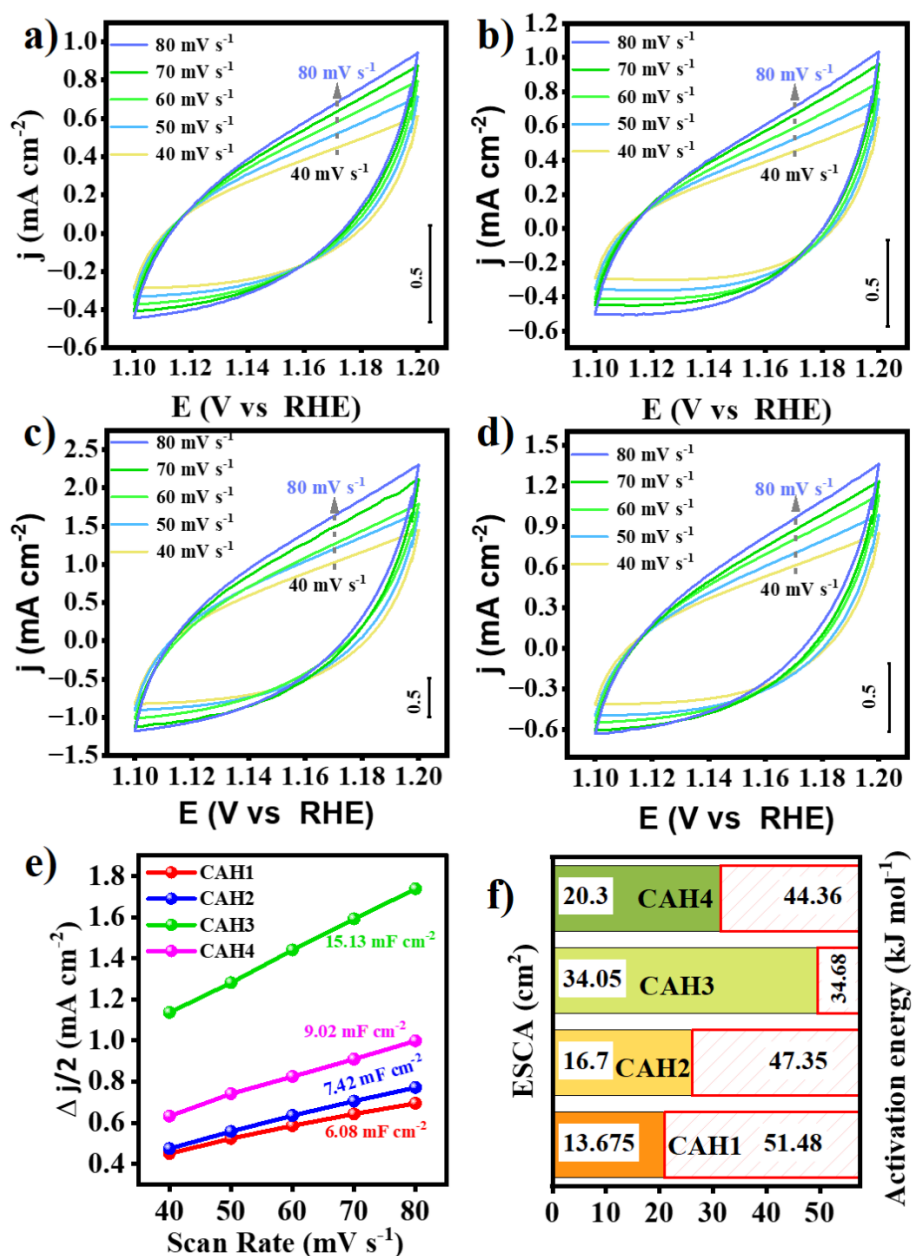


Figure 3C.7 Cyclic voltammetry obtained in a non-faradaic region at various scan rates: a) CAH1, b) CAH2, c) CAH3, and d) CAH4 in 1.0 M KOH, e) Estimation of the C_{dl} , f) ECSA and activation energy of all the catalysts.

S. No.	Catalyst code	$C_{dl}(\text{mF cm}^{-2})$	$C_{dl}(\text{mF})$	ECSA (cm ²)	RF
1	CAH1	6.08	0.547	13.675	151.90
2	CAH2	7.42	0.668	16.7	185.56
3	CAH3	15.13	1.362	34.05	378.33
4	CAH4	9.02	0.812	20.3	225.56

Table 3C.4 Summary of Double layer capacitance (C_{dl}), Electrochemical active surface area (ECSA) and Roughness factor (RF) of the studied catalysts.

Furthermore, the activation energy was calculated using the Arrhenius equation by recording LSV curves at different temperatures to understand the kinetic barrier of the water splitting reaction (**Figure 3C.8a-d**).⁴⁸ The slope of $\log j$ (mA cm^{-2}) vs. $1/T$ (K^{-1}) (**inset of Figure 3C.5a**) gives an activation energy of $34.68 \text{ kJ mol}^{-1}$ for CAH3, and $51.48 \text{ kJ mol}^{-1}$, $47.35 \text{ kJ mol}^{-1}$, and $44.36 \text{ kJ mol}^{-1}$ for CAH1, CAH2, and CAH4, respectively (**Figure 3C.7f; right axis**). This analysis shows that the reaction rate is highly dependent on temperature.

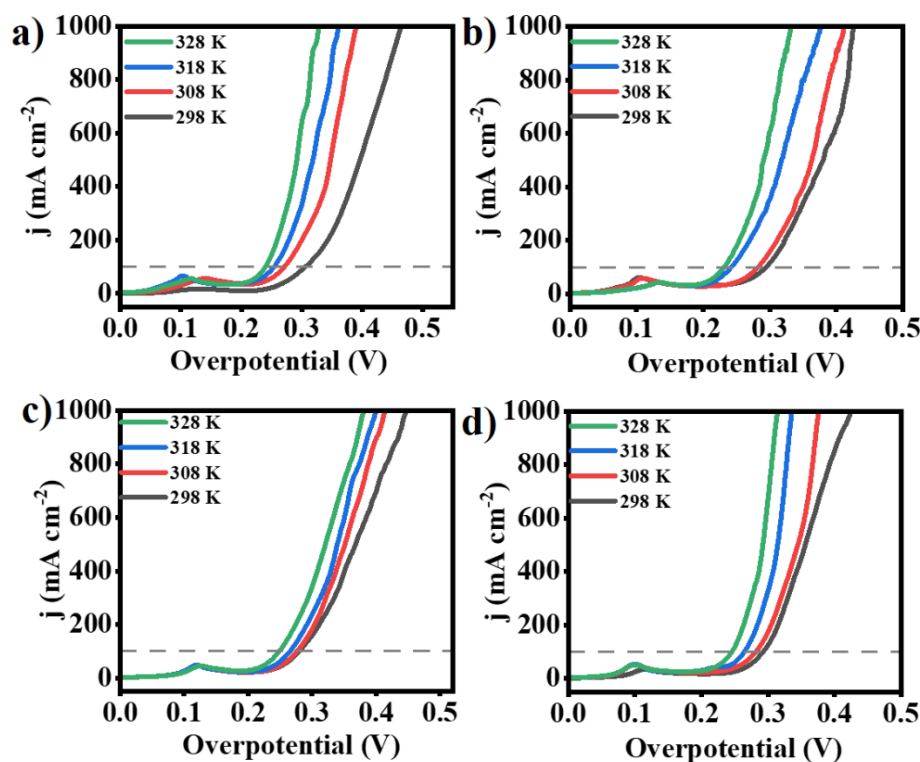


Figure 3C.8 LSV at various temperatures: a) CAH1, b) CAH2, c) CAH3, and d) CAH4 in 1.0 M KOH.

S. No.	Catalyst Code	Activation Energy, E_a (kJ mol^{-1})
1	CAH1	51.48
2	CAH2	47.35
3	CAH3	34.68
4	CAH4	44.36

Table 3C.5 Calculated Activation Energy (E_a) of all the catalysts.

Additionally, the energy barrier of the catalytic process is also lowered by the increase of polar groups in the CAH3 skeleton. Like HER, mass activity of CAH3 appeared to be maximum for OER, while the rest of the sample displayed less mass activity for an identical amount of mass loading (**Figure 3C.9a; upper panel**). Moreover, it delivered a high turnover frequency value of 0.724 s^{-1} at $\eta_{100} = 271 \text{ mV}$, indicating the high intrinsic activity of the catalytic sites for water oxidation (**Figure 3C.9a; lower panel**). During 48 hours of OER in 1.0 M KOH, CAH3 could produce 100 mA cm^{-2} current density without a discernible rise in potential, as observed in **Figure 3C.9b**.

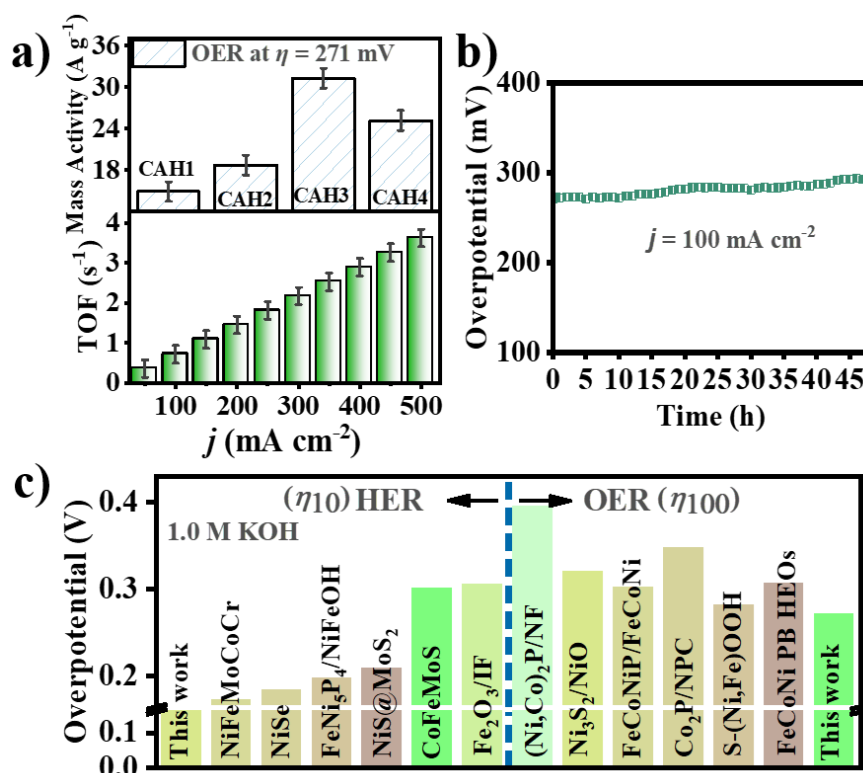


Figure 3C.9. a) Mass activity (upper) and TOF (lower) of all the samples at the given overpotential, b) Chronopotentiometry curve in the anodic region, c) Comparison of overpotentials of HER and OER with the state-of-the-art electrocatalysts in 1.0 M KOH.

Thus, CAH3 exhibited highly efficient catalytic activity for HER ($\eta_{10} = 160 \text{ mV}$) and OER ($\eta_{20} = 215 \text{ mV}$) among all the synthesized compositions, producing an impressive current density with satisfactory processability. The remarkable tunability combined with a facile

synthetic approach endows this supramolecular hydrogel catalyst with exceptional functional advantages. It outperforms most of the recently reported metal-free, polymer-based, and non-precious electrocatalysts (**Figure 3C.9c**) [47-50].

The stability of the CAH3 was evaluated after the electrocatalysis reaction. The FESEM images revealed that the fibrillar network of hydrogel remained intact after electrocatalytic HER and OER, demonstrating stability of the material even after 5000 cycles (**Figure 3C.10a, b**). The surface of the electrode was slightly exfoliated in the case of HER, which might be attributed to hydrogen embrittlement or hydrogen-induced cracking after prolonged exposure to HER conditions. The post-catalytic XPS of CAH3 suggested that cobalt remained in the +2 oxidation state (**Figure 3C.10c, d**).

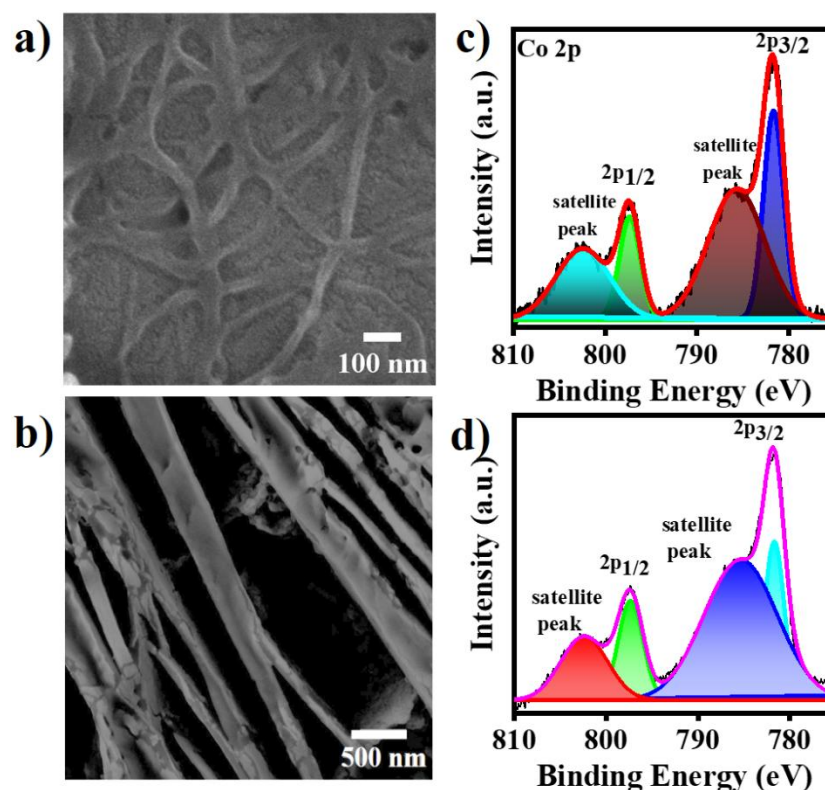


Figure 3C.10 a,b) FESEM images of CAH3 after OER and HER, respectively, c,d) Co 2p XPS survey spectrum of CAH3 after OER and HER, respectively.

3C.2.3 Electrocatalytic Overall Water Splitting Performance of CAH3

Considering the outstanding HER and OER bifunctionality performance and stability of CAH3, a full cell two-electrode arrangement of CAH3||CAH3 was assembled to act as CAH3 both as anode and cathode for overall water electrolysis in 1.0 M and 30 wt% KOH electrolytes as shown in the inset of **Figure 3C.11a**. For comparison, an electrolyzer based on precious metals (IrO₂||Pt/C) was also tested under the same experimental conditions. As shown in **Figure 3C.11a**, at room temperature, the CAH3 coupled electrolyzer showed an unexpected and exceptional performance with a cell voltage of 1.56 V at a current density of 10 mA cm⁻². It was much better than the IrO₂||Pt/C (1.62 V at 10 mA cm⁻²) as well as those of other reported state-of-the-art electrodes or even comparable to that of some advanced asymmetric and symmetric bifunctional water-splitting electrocatalysts reported recently [51-55]. Notably, the CAH3||CAH3 water electrolyzer can produce quite a high current density of over 1000 mA cm⁻². To the best of our knowledge, a nucleotide-based single metal hydrogel catalytic system capable of functioning as a complete electrolyzer for water splitting is unprecedented. Robust water electrolysis is anticipated due to the high water absorption capability of the material, which is likely to enable facile absorption and confinement of the electrolyte to the electrode surface. A direct solar-to-hydrogen conversion of the CAH3 coupled electrolyzer can also be driven under natural illumination combined with commercial silicon solar cell radiation. The electrolyzer showed effective water splitting and rapid evolution of gas bubbles and vigorous effervescence at both electrodes, endorsing the evolution of H₂ and O₂.

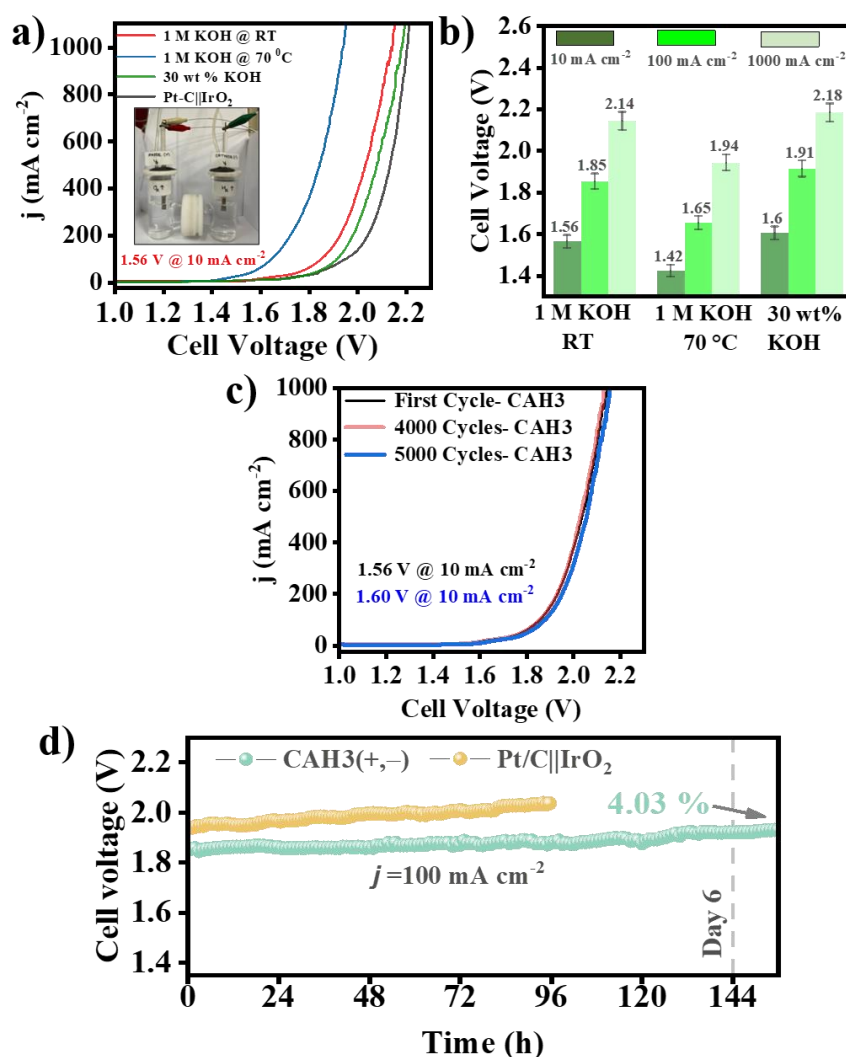


Figure 3C.11 Overall water splitting performance: a) LSV curve of CAH3||CAH3 coupled water electrolysis cell in different conditions, b) Cell voltage of the catalyst under various conditions, c) LSV curves of CAH3 before and after 5000 cycles, d) Chronopotentiometry curve of CAH3 couple electrodes at the given current density.

To expand the possibilities of industrial electrolyzer applications, the 1.0 M KOH was heated to 70 °C, which significantly reduced the cell voltage to $\eta_{10} = 1.42$ V (**Figure 3C.11a**), indicating that the activation barrier decreased upon increasing the temperature. As discussed earlier, this is due to the stability of the present catalyst across a broad temperature range, enabling its omnifunctional characteristics. Moreover, the rheological studies revealed the increase in yield point at high temperature, indicating its higher stability at elevated temperatures, thus slowing the disintegration of the hydrogel matrix (**Figure 3C.1e**). Additionally, the electrocatalytic activity of CAH3 was also evaluated

under harsh corrosive conditions (30 wt% KOH), where it achieves a current density of 10 mA cm^{-2} by executing a cell potential of 1.60 V (**Figure 3C.11a**). **Figure 3C.11b** outlines a comparison of cell voltage required to gain respective current densities under different experimental conditions.

Further, we investigated the long-term stability and durability of the CAH3||CAH3 cell using LSV cycling and chronopotentiometry test under a constant cell voltage of 1.85 V. The overlapping of the first LSV curve with the curve after 4000 cycles reveals the high operational stability and robustness of the electrolyzer system. A slight increase in the potential after 5000 cycles was observed, which might be due to rapid gas bubble accumulation on the electrode or partial detachment of the catalyst (**Figure 3C.11c**). The Chronopotentiometry test revealed that the current density of 100 mA cm^{-2} remained nearly unchanged, experiencing only a 4.03% degradation over a period exceeding 6 days (**Figure 3C.11d**). The stable multi-chronopotentiometric curve at various potentials suggests the flexible nature of the electrode, which can deal with low to high current densities up to 3 cycles with superior durability (**Figure 3C.12a**). Lastly, Faradaic efficiency (FE) was implemented to evaluate the H_2 and O_2 production during HER and OER at a constant charge supply for 180 minutes. The Faradaic efficiencies of 92.7% and 94.2% were obtained for HER and OER, which corroborate with theoretical calculated efficiencies (**Figure 3C.12b,c**). The potential of the present catalyst was assessed by comparing it with other recently reported high-performance electrocatalysts (**Figure 3C.12d**).

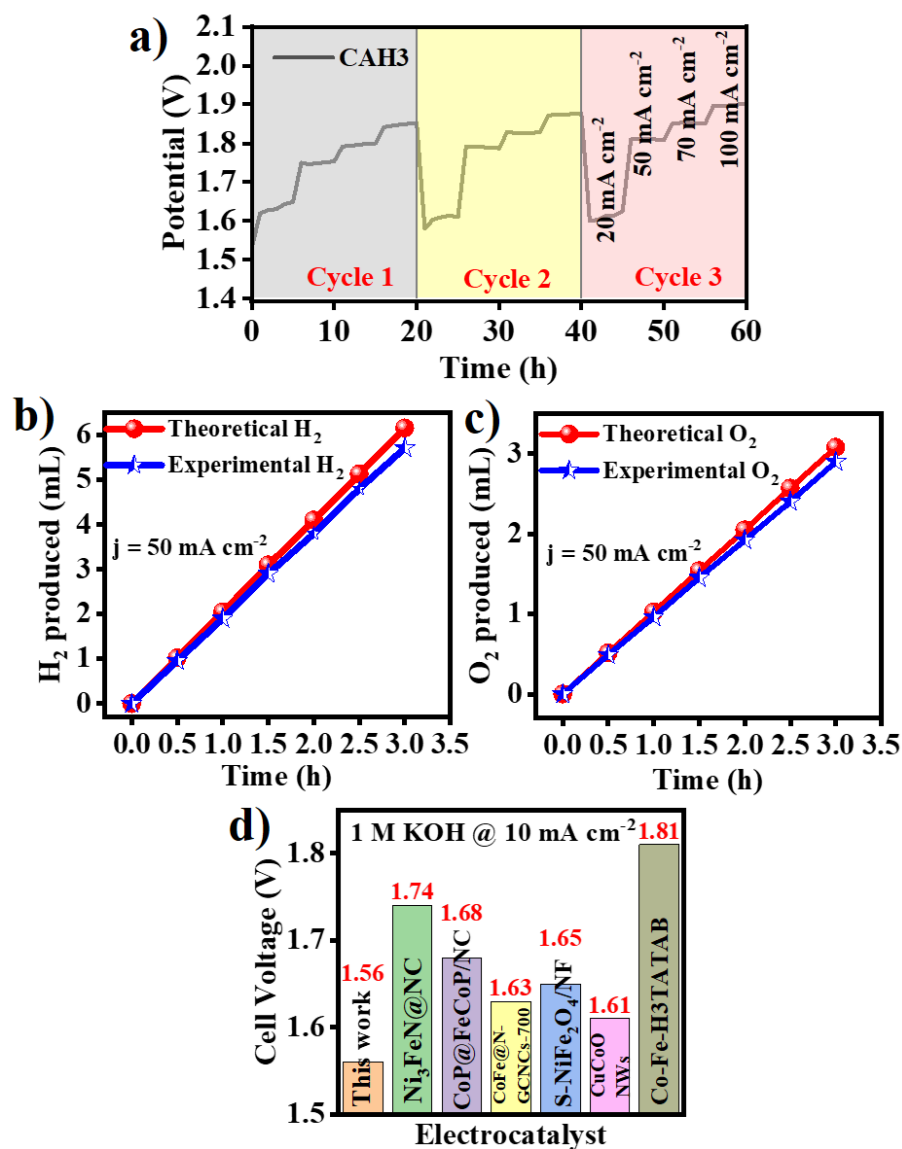


Figure 3C.12 a) Multi-chronopotentiometry curve of CAH3 catalyst, b,c) Faradaic Efficiency of HER & OER for CAH3 at an applied current density of 50 mA cm^{-2} , d) Comparison of cell voltage for overall water splitting at 10 mA cm^{-2} with recently reported robust electrocatalysts.

The ease of synthesis, prolonged stability across variable pH and temperature range coupled with high efficiency makes the Co-AMP hydrogel highly competitive for water-electrolysis. The activity was found to be comparable or even better than bimetallic and trimetallic MOF, COF, nanoparticle, and single atom based recently reported electrocatalysts, thereby contributing to the ongoing advancements in sustainable energy production. Its outstanding performance at 70°C and

30 wt% KOH is remarkable and highlights the potential of nucleotide-hydrogel for industrial implications.

3C.2.5 Mechanistic insights using DFT studies for the HER and OER activity by CAH3 hydrogel

Initially, geometry optimization of the monomer unit of CAH3 hydrogel was performed using Density Functional Theory (DFT) calculations with the B3LYP functional. The 6-31G basis set was employed for carbon (C), hydrogen (H), nitrogen (N), oxygen (O), phosphorus (P), and chlorine (Cl) atoms, while the LANL2DZ basis set with effective core potentials (ECPs) was used for the cobalt (Co) atom. The structure, shown in **Figure 3C.13**, is stabilized by hydrogen bonding between the N7 and NH₂ group of adenine base residue and the phosphate group of AMP. Additionally, the sugar moiety also participates in hydrogen bonding with the phosphate group, further contributing to structural stability.

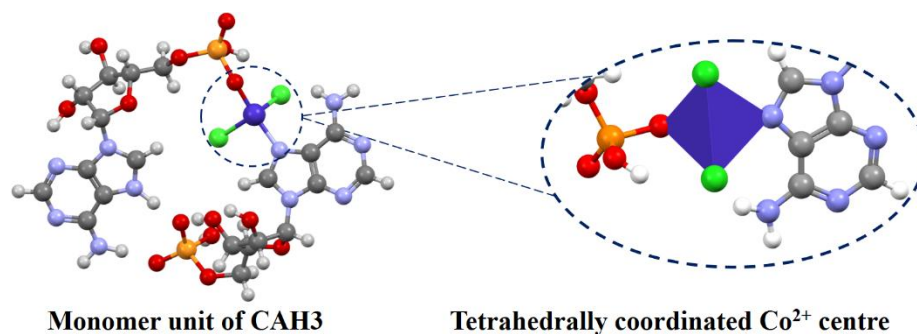


Figure 3C.13 Geometry optimized structure of monomer unit of CAH3 hydrogel.

	Bond Angle (rad)		Bond Length (Å)
Cl1-Co-O	101.54	CoCl1	2.22
Cl1-Co-N	94.02	Co-Cl2	2.21
Cl1-Co-Cl2	100.52	Co-O	1.74
Cl2-Co-O	100.31	Co-N	1.98
Cl2-Co-N	96.43		
O-Co-N	154.54		

Table 3C.6 The calculated bond angles and bond lengths of the optimized monomer unit of CAH3.

The bond lengths of Co–O, Co–N, Co–Cl1, and Co–Cl2, along with their respective bond angles, are summarized in **Table 3C.6**.

DFT calculations were performed to gain molecular-level insights into the HER and OER activity of the hydrogel. All intermediate structures were optimized and verified to exhibit no imaginary frequencies, ensuring their stability. The HER pathway was investigated using CAH3, with all the structures optimized via the B3LYP DFT method. The study commenced with the structural optimization of CAH3, considering cobalt as the active center. The initial step involves the coordination of a water molecule, leading to the formation of the CAH3–H₂O complex. The calculated water adsorption energy of –0.91 eV indicates a stable penta-coordinated structure. Subsequently, the H–OH bond in the adsorbed water weakens due to the strong interaction between the hydroxyl group and the P=O moiety of the phosphate group in AMP, facilitating the removal of OH[–] and thus leading to a more stable CAH3–H complex ($E_{\text{ad}} = -1.02$ eV). Subsequent adsorption of a second water molecule yields a hexa-coordinated CAH3–H·H₂O complex ($E_{\text{ad}} = -1.80$ eV), wherein the oxygen atom of the phosphate group continues to play a crucial role by further weakening the H–OH bond. This enables the removal of OH[–] and conversion to the CAH3–H₂ complex ($E_{\text{ad}} = -1.32$ eV). In the final stage, one hydrogen atom remains bound to the cobalt center, while the other is stabilized with the oxygen of the phosphate group (**Figure 3C.14a,c**). These results underscore the crucial role of the phosphate moiety of the ligand (AMP) not only in stabilizing the key reaction intermediates but also in mediating essential bond activation steps. In this context, such ligand-assisted mechanisms can be an effective strategy to design efficient electrocatalysts.

Further, the mechanistic details for the OER were investigated. The OER on the cobalt complex CAH3 proceeds through a series of well-defined steps involving sequential hydroxide ion adsorption and changes in the oxidation state of cobalt. The process initiates with the adsorption of a hydroxide ion (OH[–]) at the cobalt center, forming the CAH3–OH intermediate with an adsorption energy of –3.67 eV.

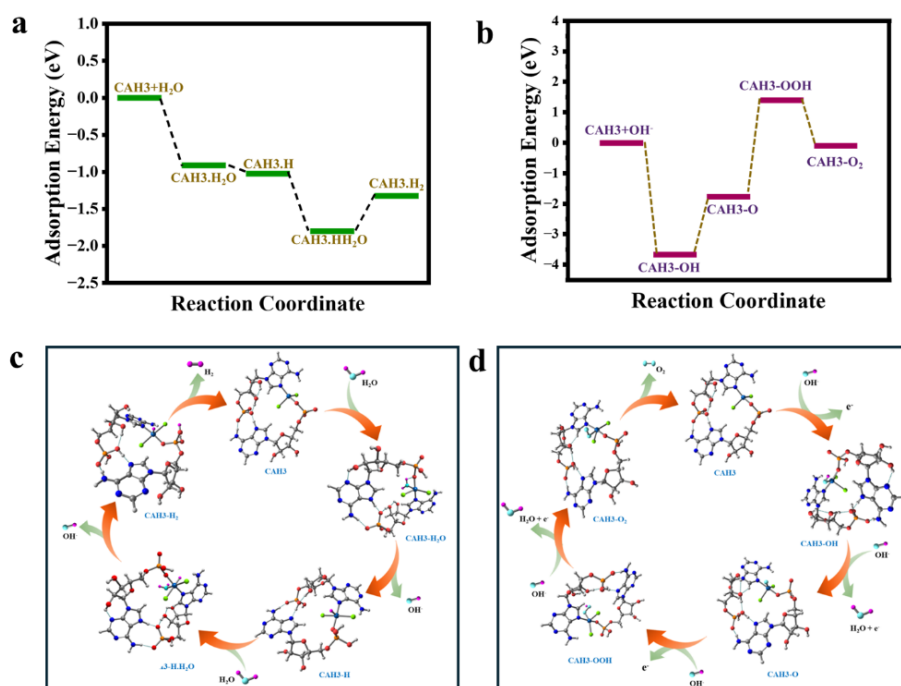


Figure 3C.14 a,b) The relative adsorption energy profile diagrams for HER and OER, respectively; c,d) Schematic representations of the proposed reaction pathways for HER and OER, respectively. All intermediate structures involved in the HER and OER reactions were optimized using DFT.

This step is accompanied by an electron removal and oxidizing cobalt from +2 to +3, resulting in Co–O and O–H bond lengths of 1.82 Å and 0.98 Å, respectively. In the next step, a second OH^- ion interacts with the $\text{CAH3} \cdot \text{OH}$ complex to form $\text{CAH3} \cdot \text{O}$ intermediate in a thermodynamically favorable manner ($E_{\text{ad}} = -1.77$ eV). In this process, cobalt is oxidized to +4, and the Co–O bond is shortened to 1.74 Å. The addition of a third OH^- leads to the formation of the $\text{CAH3} \cdot \text{OOH}$ which is a key intermediate in the OER process with an adsorption energy of 1.40 eV, with characteristic Co–O, O–O, and O–H bond lengths of 1.95 Å, 1.40 Å, and 0.99 Å, respectively. This step is accompanied by an electron transfer, which reduces the central cobalt ion back to +3 oxidation state. Subsequently, a fourth OH^- ion adsorbs, producing $\text{CAH3} \cdot \text{O}_2$ ($E_{\text{ad}} = -0.10$ eV), with Co–O and O–O bond lengths of 2.05 Å and 1.26 Å, and the cobalt center returning to its original +2 oxidation state. Finally, molecular oxygen (O_2) is released from the $\text{CAH3} \cdot \text{O}_2$ complex, regenerating the original CAH3 catalyst and completing the catalytic cycle (Figure 3C.14b,d).

This stepwise mechanism highlights the crucial role of the flexible oxidation states of cobalt and the structural adaptability in enabling efficient OER catalysis. Although low coordination numbers in transition metal complexes are typically associated with instability, the stable and sustained performance of the four/five-coordination of cobalt is remarkable [33]. The structural stability and flexibility indicate the important interplay between the cobalt centre and phosphate polyanion framework. Furthermore, the theoretical overpotential was calculated to be 246 mV as estimated from the rate-limiting step involving the transformation of CAH3-OOH to CAH3-O₂. These results clearly highlight the synergistic role of the phosphate ligand in concert with the metal centre in enhancing the electrochemical efficacy of the hydrogel, which corroborates well with the experimental results and explains the superior electrocatalytic activity of CAH3 among other hydrogel samples.

3C.3 Conclusion

In summary, we utilized a supramolecular heat-set Co-AMP hydrogel for electrochemical HER, OER, and overall water splitting. CAH3 (1:2 Co-AMP hydrogel) was screened as the best electrocatalyst among the other hydrogel samples. The hydrogel showed a low cell voltage of 1.56 V for overall water splitting to achieve a current density of 10 mA cm⁻² in alkaline conditions (1 M KOH) at room temperature. At 70 °C, it showed outstanding electrocatalytic performance with a cell voltage of 1.42 V in 1 M KOH, while under corrosive conditions (30 wt% KOH), the cell voltage was 1.60 V, hydrogel, outperforming most of the benchmark catalytic systems. These findings also highlight the potential of the material as an efficient electrocatalyst in industrial conditions. This structural integration, including the phosphate group, tetrahedral coordination around the cobalt centre, and the hydrogel character of the material, altogether enabled the faster reaction kinetics resulting in the enhanced and efficient electrocatalytic performance. Its facile synthetic methodology, long-term stability in ambient conditions, ease of electrode fabrication, thixotropic and swelling behaviour, make

the nucleotide-based hydrogel an efficient catalytic system for the clean and sustainable energy production. Further, the DFT calculations also suggested the synergistic role of phosphate as a ligand in enhancing the electrochemical efficacy of the hydrogel. These findings lay the foundation for a new paradigm in the rational design of efficient bifunctional electrocatalysts, uniting synthetic simplicity with high catalytic efficiencies.

3C.4 Experimental Section

Materials and Methods

Materials

Adenosine 5'-monophosphate disodium salt (AMP- Na_2), Cobalt Chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), and Water HPLC grade were purchased from Sisco Research Laboratory, India (SRL). Ethylenediaminetetraacetic acid disodium salt dihydrate, Platinum carbon (Pt/C, 20 wt%), and Iridium oxide (IrO_2) were purchased from Sigma-Aldrich, USA. Potassium hydroxide (KOH) and Nafion binder (5 wt%) were obtained from Alfa Aesar. All the chemicals were used as received without further purification.

Electrochemical Measurements

The electrochemical measurements, i.e., Linear Sweep Voltammetry (LSV), Cyclic Voltammetry (CV), Electrochemical Impedance Spectroscopy (EIS), and Chronopotentiometry (CP) on the laboratory scale for both the half-cell reactions (Hydrogen Evolution Reaction (HER) & Oxygen Evolution Reaction (OER)) were performed at STP in a conventional three-electrode setup using a CHI660E electrochemical workstation. In the three-electrode configuration, an Hg/HgO electrode (saturated with 1.0 M KOH) and a graphite rod were operated as the reference and the counter electrode, respectively. In contrast, the working electrode was prepared by depositing catalyst ink onto the commercially available Nickel foam substrate. The overall water splitting system was fabricated by arranging a full cell of two electrode

configurations in an H-cell utilizing CAH3/NF bi-functional catalyst as the anode and cathode. In the H-cell, a Nafion membrane served as the separator.

Before drop-casting of the catalyst, the NF substrate was ultrasonically cleaned with 30% HCl, water, and ethanol to remove surface impurities. To prepare a homogeneous catalyst ink, 10 mg of catalyst was dispersed in 0.5 mL of absolute ethanol, and 20 μL of 5% Nafion binder solution was added by ultrasonication for about half an hour. Then, 15 μL of as-prepared catalyst ink was firmly coated over the nickel foam substrate of an area of 0.09 cm^2 and allowed to dry overnight at ambient conditions. An optimum mass loading amount of 3.2 mg cm^{-2} was ensured for the electrochemical studies.

All electrochemical potentials were calibrated relative to the reversible hydrogen electrode (RHE) according to the Nernst Equation:

$$E_{\text{RHE}} = E_{\text{Hg/HgO}} + E^0_{\text{Hg/HgO}} + 0.059 \times \text{pH} \quad (1)$$

$$(E^0_{\text{Hg/HgO}} = 0.098 \text{ at } 25^\circ\text{C and pH} = 13.6 \text{ for } 1.0 \text{ M KOH})$$

Additionally, all potentials of polarization curves were iR corrected unless otherwise stated to account for the impact of uncompensated solution resistance (R_s) using the following equations to deal with the catalyst's real potential:

$$E_{\text{actual}} = E_{\text{applied}} - iR_s \quad (2)$$

The HER and OER overpotentials (η) were calculated at different current densities (x) using equation S3:

$$\eta_x = (E_{\text{RHE}} - Z) \times 1000 \text{ (in mV)} \quad (3)$$

where Z denotes the thermodynamic potentials for HER (0 V) and OER (1.23 V).

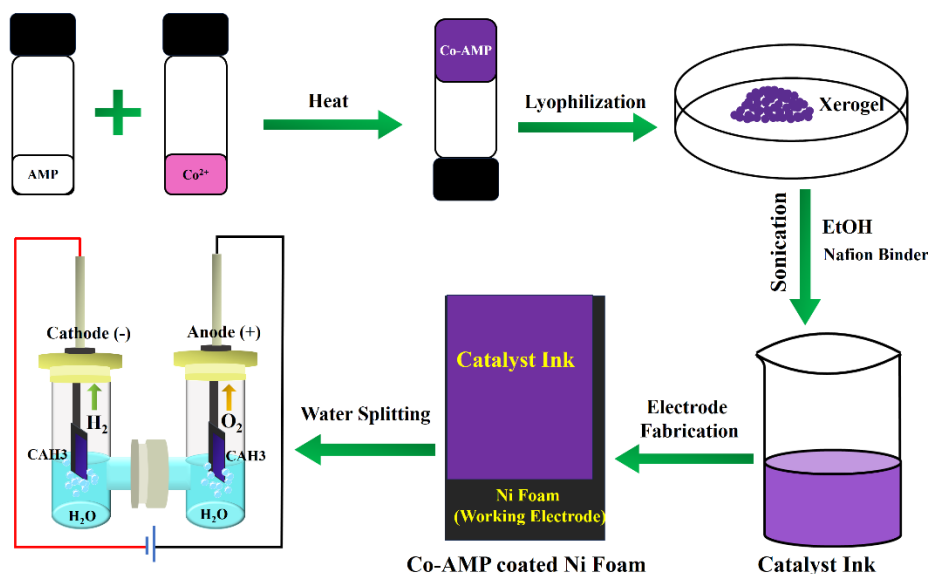
Before performing the electrochemical measurement, 1.0 M KOH and 30 wt% KOH were saturated with N_2 and taken as alkaline electrolytes. To activate the electrocatalyst, cyclic voltammetry at higher

anodic and cathodic potentials was conducted at a scan rate of 100 mV s⁻¹ before investigating the HER and OER studies. The LSV polarization curves were recorded at a lower potential sweep of 10 mV s⁻¹ to lessen the capacitive current. The potential window for HER and OER is 0.0 to -0.8 V (vs RHE) and 1.0 to 1.8 V (vs RHE), respectively. Electrochemical impedance spectroscopy measurements were executed to determine the charge transfer resistance by an AC voltage of 100 mV amplitude within a frequency range of 100 kHz to 0.1 Hz with bias potential of -0.18 V and 1.53 V (vs RHE) for HER and OER, respectively. The CV curves at various scan rates in a non-faradaic region were used to estimate the double-layer capacitance (C_{dl}), from which the electrochemical active surface area (ECSA) was calculated. An Eudiometry test was employed to determine the Faradaic efficiency by measuring the volume of water displaced in an inverted burette, where the displacement directly correlated with the quantity of gas generated at the working electrode. Stability and durability tests were done for the best-performing CAH3 catalyst at different current densities.

The Tafel plots were determined by fitting the polarization curve to the Tafel equations:

$$\eta = a + b \log j \quad (4)$$

(where 'η' is the overpotential, 'j' is the current density, 'b' is the Tafel slope, and 'a' is a constant).



Scheme 3C.1 Graphical representation for the electrode fabrication using CAH xerogels for electrochemical measurements.

Calculations:

1. Exchange current density:

$$\text{Exchange current density } (i_{ex}) = \frac{RT}{nFR_{ct}A} \quad (5)$$

Where,

R: Universal gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$),

T: Reaction temperature (298 K),

n: Number of electrons: $n = 2$ (HER); $n = 4$ (OER),

F: Faraday constant (96485 C mol^{-1}),

R_{ct} : Charge transfer resistance calculated from EIS,

A: Area of the loaded catalyst on Nickel foam (0.09 cm^2)

(a) For HER in 1 M KOH (2 electron process)

$$\begin{aligned} &= \frac{8.314 \text{ J K}^{-1} \text{ mol}^{-1} \times 298 \text{ K}}{2 \times 96485 \text{ C mol}^{-1} \times 6.69 \Omega \times 0.09 \text{ cm}^2} \\ &= 21.32 \text{ mA cm}^{-2} \end{aligned}$$

(b) For OER in 1 M KOH (4 electron process)

$$= \frac{8.314 \text{ J K}^{-1} \text{ mol}^{-1} \times 298 \text{ K}}{4 \times 96485 \text{ C mol}^{-1} \times 4.57 \Omega \times 0.09 \text{ cm}^2}$$

$$= 15.6 \text{ mA cm}^{-2}$$

2. Mass activity:

Mass activity (A/g) = j/m ; at $\eta = 275 \text{ mV}$ (HER) and $\eta = 271 \text{ mV}$ (OER)

(6)

Where,

j : Current density (mA cm^{-2});

m : mass loading onto the nickel foam working electrode (mg cm^{-2})

$$= \frac{100 \text{ mA cm}^{-2}}{3.2 \text{ mg cm}^{-2}} = 31.25 \text{ mA cm}^{-2}$$

3. Electrochemical active surface area (ECSA):

$$\text{ECSA} = C_{\text{dl}} (\text{mF}) / C_s (\text{mF cm}^{-2}) \quad (7)$$

Where, C_{dl} = double layer capacitance of the electrode in 1.0 M KOH and C_s = specific capacitance of the surface (For Ni foam, 0.04 mF cm^{-2} in 1.0 M KOH).

The double layer capacitance (C_{dl}) was calculated from the slope of current density vs. scan rates of cyclic voltammetry obtained in a non-faradaic region. Thus,

$$C_{\text{dl}} (\text{F cm}^{-2}) =$$

Slope of the plot of $\Delta j/2$ (mA cm^{-2}) vs scan rate (mV s^{-1})

Geometrical surface area of the electrode used = $0.3 \times 0.3 \text{ cm}^2$

$$C_{\text{dl}} (\text{mF}) = C_{\text{dl}} (\text{mF cm}^{-2}) \times \text{electrode area (cm}^2)$$

$$= 15.13 \text{ mF cm}^{-2} \times 0.09 \text{ cm}^2 = 1.362 \text{ mF}$$

$$\text{ECSA (cm}^2) = \frac{1.362 \text{ mF}}{0.04 \text{ mF cm}^{-2}}$$

$$= 34.05 \text{ cm}^2$$

4. Roughness Factor (RF):

$$\text{Roughness factor (RF)} = \text{ECSA}/A \quad (8)$$

Where A is the area of the loaded catalyst on Ni Foam (0.09 cm^2)

$$\begin{aligned} \text{RF in case of HER} &= \frac{34.05 \text{ cm}^2}{0.09 \text{ cm}^2} \\ &= 378.3 \end{aligned}$$

5. Turn over frequency (TOF) calculation:

$$\text{Turn over frequency (TOF)} = \frac{j \times A}{n \times F \times n_s} \quad (9)$$

Where, j: Current density (mA cm^{-2}),

A: Area of the electrode (cm^2)

n: number electrons involved in the process, F: Faraday constant (96485 C mol^{-1})

N_s : Concentration of the active sites in the catalyst (mol cm^{-2})

To calculate N_s , cyclic voltammetry (CV) was carried out in a non-Faradaic region at different

scan rates in 1.0 M KOH electrolytes. The peak currents were plotted versus scan rates where

the slope is equal to

$$\text{Slope} = \frac{F^2 A N_s}{nRT}$$

Where n: number of electrons transferred, F: Faradaic constant, A: surface area of the electrode, R: universal gas constant, and T: absolute temperature.

$$N_s = 1.61 \times 10^{-8} \text{ mol (for HER)}$$

$$N_s = 3.22 \times 10^{-8} \text{ mol (for OER)}$$

$$\begin{aligned} \text{TOF (for HER)} &= \frac{100 \text{ mA cm}^{-2} \times 0.09 \text{ cm}^2}{2 \times 96485 \text{ C mol}^{-1} \times 1.61 \times 10^{-8} \text{ mol}} \\ &= 2.89 \text{ s}^{-1} \end{aligned}$$

$$\begin{aligned} \text{TOF (for OER)} &= \frac{100 \text{ mA cm}^{-2} \times 0.09 \text{ cm}^2}{4 \times 96485 \text{ C mol}^{-1} \times 3.22 \times 10^{-8} \text{ mol}} \\ &= 0.724 \text{ s}^{-1} \end{aligned}$$

6. Activation energy calculation (E_a):

Arrhenius equation was used to calculate the activation energy (E_a) by plotting $\log j$ against the inverse of temperature.

$$\frac{\partial (\log j)}{\partial (1/T)} = \frac{-E_a}{2.303 R} \quad (10)$$

Where $\frac{\partial (\log j)}{\partial (1/T)}$ is the slope from the inset of Figure 3C.5a. For CAH3

$$\frac{\partial (\log j)}{\partial (1/T)} = -1811 \text{ K}$$

$$\text{Thus, } E_a = 1811 \times 2.303 \times 8.314 \text{ J. K}^{-1}\text{mol}^{-1}$$

$$= 34675.47 \text{ Jmol}^{-1}$$

$$= 34.68 \text{ kJ. mol}^{-1}$$

7. Faradaic efficiency measurement and quantification of H_2 and O_2 gas evolved for CAH3 catalyst:

The theoretical amount of total gas volume expected was determined using Faraday's law of electrolysis with the ideal gas law:

$$V_{theo} = \frac{RT}{F_{pz}} Q \quad (11)$$

Where, R: ideal gas constant, T: absolute temperature, Q: charge (at an applied current density of 50 mA cm^{-2} for 180 minutes), F: Faraday constant, p: pressure and z: number electrons involved.

The faradaic efficiency (ηF) of the water electrolysis process was monitored by comparing the actual measured gas volume to the theoretical total gas volume expected.

$$\eta F = \frac{V_{meas}}{V_{theo}} \quad (12)$$

Where, V_{meas} is the measured H_2 and O_2 gas volume using the inverted glass burette method for the HER and OER process, respectively.

The faradaic efficiency of H_2 gas evolution is:

$$\eta F = \frac{5.71}{6.16} \times 100 = 92.7\%$$

The faradaic efficiency of O_2 gas evolution is:

$$\eta F = \frac{2.9}{3.08} \times 100 = 94.2\%$$

DFT Calculations

We have optimized the structure of the cobalt-AMP complex with Density functional theory (DFT) calculations by using Gaussian 09. We have used the B3LYP basis function for both the Hydrogen evolution reaction (HER) and Oxygen evolution reaction (OER) evaluations. We have used the 6-31G basis set for the Carbon (C), Hydrogen (H), Nitrogen (N), Oxygen (O), Phosphorous (P), Chlorine (Cl), and LANL2DZ basis set with Effective Core Potentials (ECPs) for the Cobalt (Co) atom for the optimization of the cobalt-complex. The DFT-B3LYP method is found to provide reliable energy barriers in both the chemical reaction HER and OER mechanisms. Therefore, the B3LYP method has been used to explore the HER and OER mechanisms with the reaction pathways on the active surface of the CAH3 by calculating the free energy changes of the different reaction intermediates and the energy barriers during the HER and OER reaction

process. Herein, we have used the ChemCraft visualization software to visualize and analyse all the equilibrium intermediates and electronic molecular model structures resulting from the HER and OER process.

3C.5 References

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Chapter 4

Glutathione-Nucleotide Supramolecular Hydrogels with Intrinsic Antiviral, Antibacterial, and Anticancer Activities

4.1 Introduction

The inherent propensity of peptides to self-assemble into a diverse array of nanostructures positions them as ideal building blocks for the fabrication of higher-order supramolecular hydrogels [1-4]. Designing peptide-based hydrogels presents a compelling strategy for developing biocompatible materials with broad potential across biomedical applications [5-9]. Short peptides are particularly attractive candidates for constructing hierarchical nanoarchitectures due to their facile synthesis, intrinsic biocompatibility, and the exploration of the underlying fundamental interactions underpinning complex self-assembly processes [10,11]. The formation of advanced superstructures, such as fibrils capable of entrapping substantial volumes of water within hydrogels, critically depends on the presence of aromatic groups and hydrogen-bonding motifs. Accordingly, short peptides are often chemically modified to incorporate aromatic groups that promote π - π stacking and directional non-covalent interactions [6,12,13]. Although several aliphatic peptides exhibit notable biological activity, chemical modifications required for hydrogelation may compromise their native functionalities.

Glutathione (GSH), a naturally occurring tripeptide composed of cysteine, glycine, and glutamate, is among the most potent endogenous antioxidants. It plays a central role in maintaining redox homeostasis and protecting against oxidative stress. Additionally, GSH is involved in mitochondrial maintenance, cellular proliferation, regeneration of vitamins C and E, enhancement of insulin sensitivity, immune

modulation, and regulation of programmed cell death. The cellular redox state, governed by the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), is vital for gene regulation, redox signaling, and metabolic control [14-16]. However, being a low molecular weight antioxidant, it suffers from poor thermal stability. To overcome this limitation, it can be covalently crosslinked to a polymer matrix or conjugated with other moieties, but this crosslinking and conjugation can alter their inherent features [17]. An alternative approach is to exploit its self-assembling capabilities to construct supramolecular hydrogels, thereby preserving its native structure and bioactivity. Guanosine monophosphate (GMP) holds substantial interest among all other nucleotides as it is capable of forming several types of G-assembly like dimers, G-quartet, sheets, ribbons, etc., by taking advantage of both Watson–Crick and Hoogsteen faces in hydrogen bonding [18-24]. Taking all the factors into account, we employed guanosine monophosphate (GMP) as a low molecular weight gelator (LMWG) to co-assemble with GSH and GSSG.

Pathogenic microbes, such as bacteria and viruses, are responsible for causing several diseases affecting human life worldwide. The rising threat of antimicrobial resistance further complicates the treatment and limits the effectiveness of available antibiotics [25,26]. Hence, it is crucial to develop new formulations and alternatives to address these challenges. The recent outbreak of COVID-19 also highlighted the urgency to develop innovative alternatives to prevent the transmission and spread of pathogens to minimize health hazards [27]. The advancement of antimicrobial coatings on surfaces, masks, and other medical equipment is essential to mitigate the spread of pathogens [28]. Cancer is also posing a serious threat to human health, ranking among the leading causes of death worldwide [29,30]. To develop anticancer drugs with minimal side effects is one of the major concerns. Thus, the quest to develop biomaterials with multimodal bioactivities is the need of the hour [31-33]. Since reduced glutathione levels are associated with many diseases and known to possess inherent

antibacterial and antiviral activity, it prompted us to develop a glutathione-based hydrogel where the biochemical properties of glutathione can be amalgamated with the unique characteristics of hydrogel [34].

Herein, the development of two hydrogels obtained from the self-assembly of GSH and GSSG with GMP by simple one-pot-mixing of two components under ambient conditions is reported. The gelation is facilitated by the formation of a G-quadruplex and subsequent non-covalent interactions. The hydrogels were stable in a wide pH window from 1 to 12 at room temperature and can be stored in their xerogel form for a long time. The obtained hydrogels displayed thermo-reversibility, self-healing, and shape-sustaining behaviour. Both the obtained gels are mechanically stable, thixotropic, and injectable. These supramolecular hydrogels showed excellent biocompatibility and demonstrated *in vitro* antiviral activity against EBV and SARS-CoV-2, antibacterial activity against both gram-positive (*B. subtilis*) and gram-negative (*E. coli*) bacteria, and potent anticancer activity in gastric epithelial cells. The facile synthetic methodology using pristine biomolecules devoid of any covalent modifications can substantially impact the application of hydrogels for biological and therapeutic purposes.

4.2 Results and Discussion

4.2.1 Synthesis and Physical Properties of Hydrogels

A self-assembled hydrogel was obtained when an aqueous solution of GMP (200 mM) was mixed with an aqueous solution of GSH (200 mM), followed by slight heating to 50 °C and cooling to room temperature (GG1). Surprisingly, the addition of an aqueous solution of oxidized glutathione (GSSG) with GMP in an equimolar ratio of 1:1 (200 mM), following a similar procedure, also resulted in a highly stable hydrogel (GG2). The visual confirmation of gelation was evident from the inverse vial test (**Figure 4.1a, 4.1b**). A concentration-dependent study showed that the minimum gelation concentration for GSH and GMP was 80 mM and 90 mM, respectively, for GG1 gel (**Figure 4.1c**).

In the case of GG2 hydrogel, the minimum gelation concentration was relatively lower and found to be 70 mM for GSSG and 50 mM for GMP (Figure 4.1d). Therefore, all further studies were performed with a 1:1 molar ratio of GSH or GSSG and GMP (100 mM each) in the case of the hydrogels.

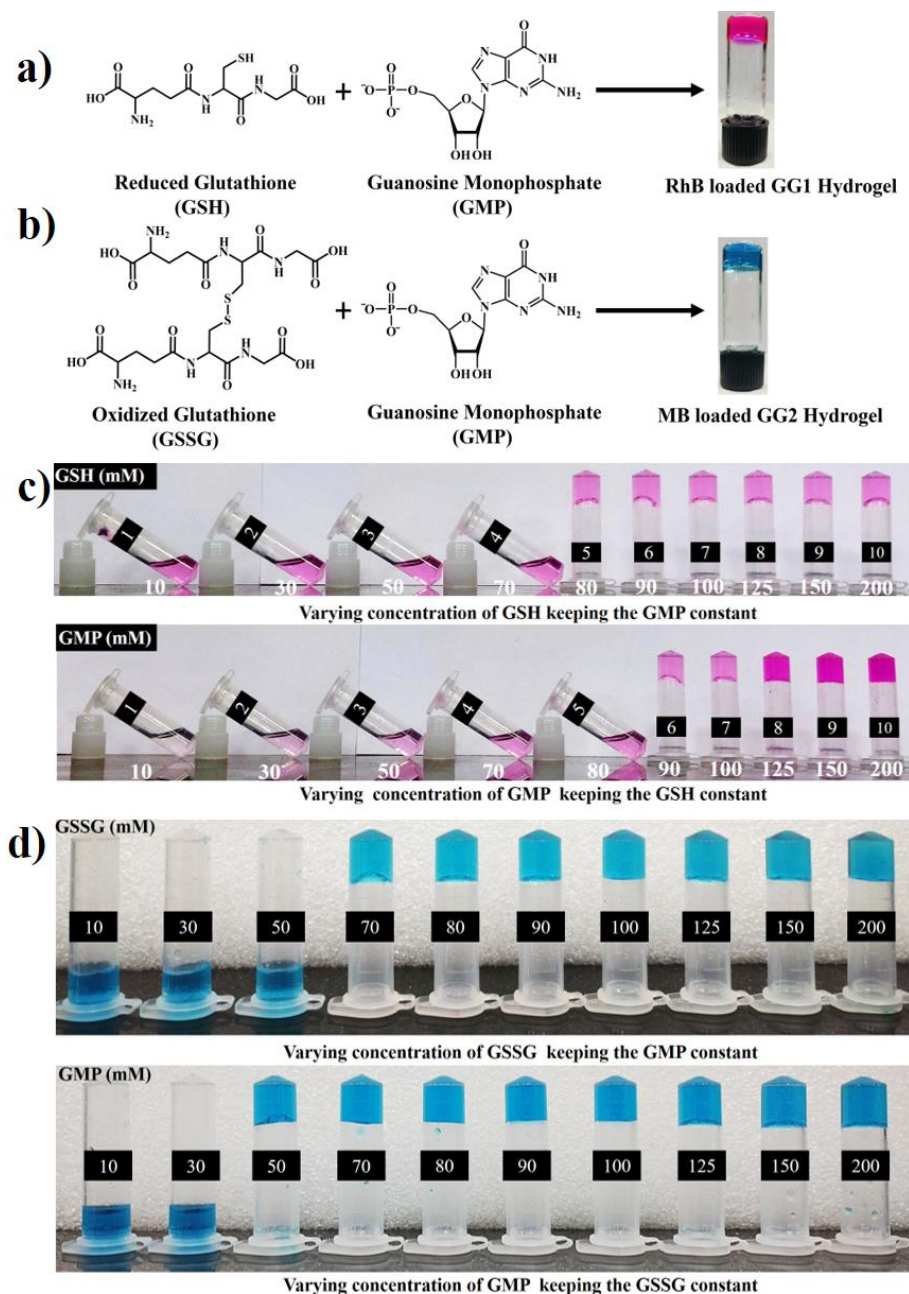


Figure 4.1 a,b) Schematic representation for the synthesis of GG1 and GG2 hydrogels, c,d) Digital images showing the minimum gelation concentration and effect of concentration on gelation with respect to both the components for GG1 and GG2 hydrogels, respectively.

Both GSH and GSSG formed hydrogels with GMP only, and no gelation occurred with other nucleotides, such as adenosine monophosphate (AMP), cytosine monophosphate (CMP), and inosine monophosphate (IMP) (**Figure 4.2a**). Both GG1 and GG2 hydrogels were thermo-responsive and could be converted to sol when heated at a temperature above 50 °C. The gel state can be restored when it is cooled to room temperature (**Figure 4.2b**). This sol-gel transition can be repeated over several heating-cooling cycles, depicting the thermo-reversible nature of the hydrogels. Taking advantage of this thermo-reversible behaviour, these hydrogels could be cast into different shapes, depicting the shape-sustaining property of the hydrogels (**Figure 4.2c**).

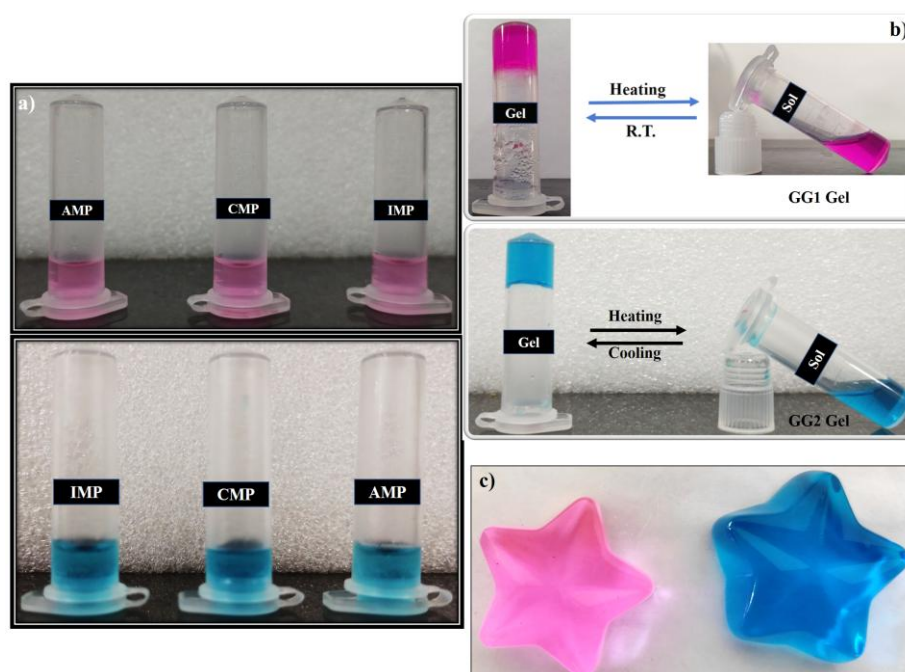


Figure 4.2 Digital images depicting a) Specific gelation of both hydrogels with GMP among other nucleotides, b) Thermo-reversible property of GG1 and GG2 hydrogels, c) Shape-sustaining behaviour of GG1 and GG2 hydrogels.

Further, the injectable and printable property of the hydrogels was investigated by extruding both the hydrogels from a needle. The gel maintains its shape when it is injected into the phosphate buffer (pH 7.4) with the help of a needle. Both the hydrogels maintained their gel state and shape even after extrusion from the needle, indicating their stability (**Figure 4.3a, 4.3b**). Further, both GG1 and GG2 hydrogels can be used

to write by extruding the hydrogel from the syringe, depicting the potential utilization of both gels for bioprinting applications (**Figure 4.3c**). Hydrogels formed via non-covalent interactions often display self-healing behaviour. To investigate the self-healing property of these hydrogels, they were dissected into two rectangular pieces and then placed together. After a few hours, the gel self-healed and restored to their original shape, and can be suspended in the air by holding from one side (**Figure 4.3d**). This observation suggested the dynamic nature of self-assembly.

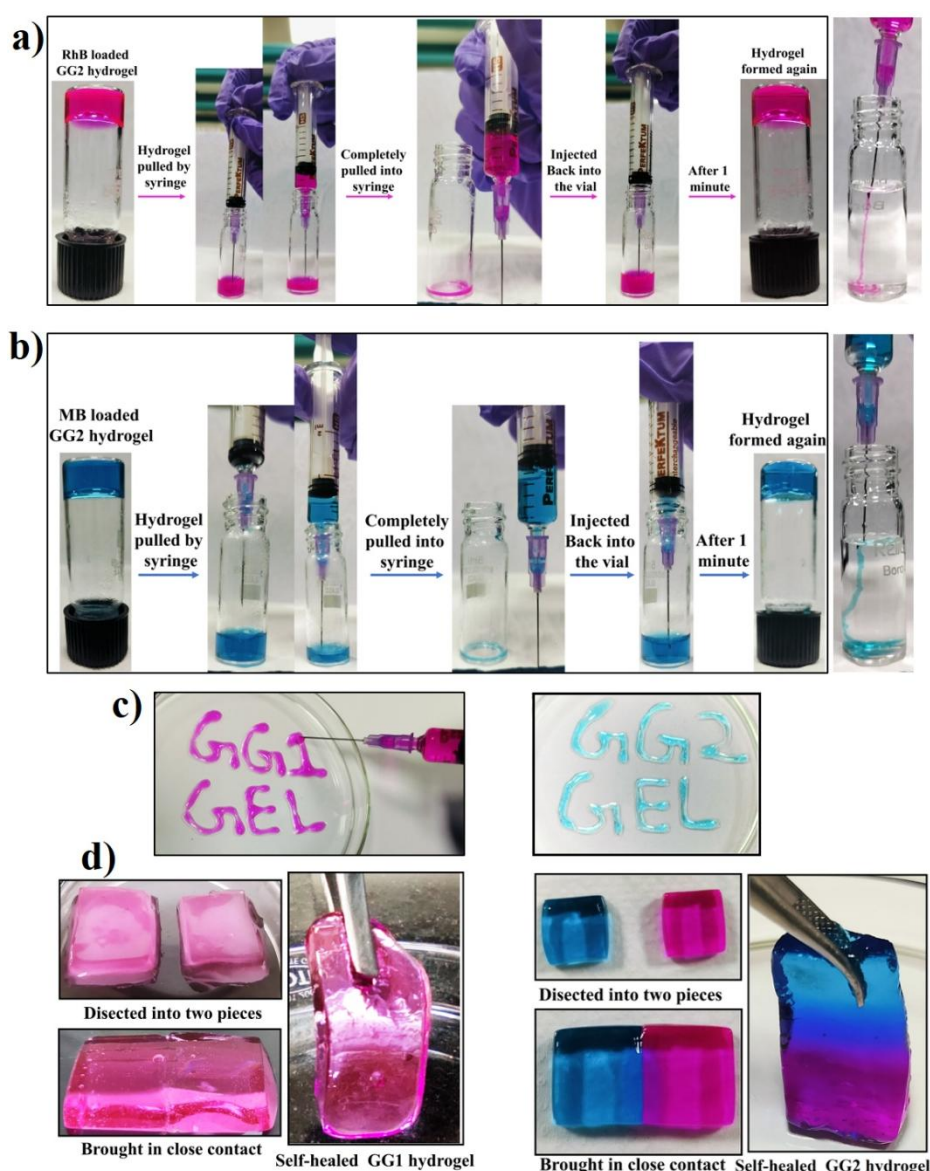


Figure 4.3 Digital images showing: a,b) Injectable and thixotropic behaviour of GG1 and GG2 hydrogels, respectively, c) Printability of hydrogels, d) Self-healing property of both the hydrogels.

The effect of pH on the gel formation was also studied, and it was observed that the gel formation took place in the pH range of 1-12 (**Figure 4.4**). This indicated that the gel was highly stable in a wide pH window.

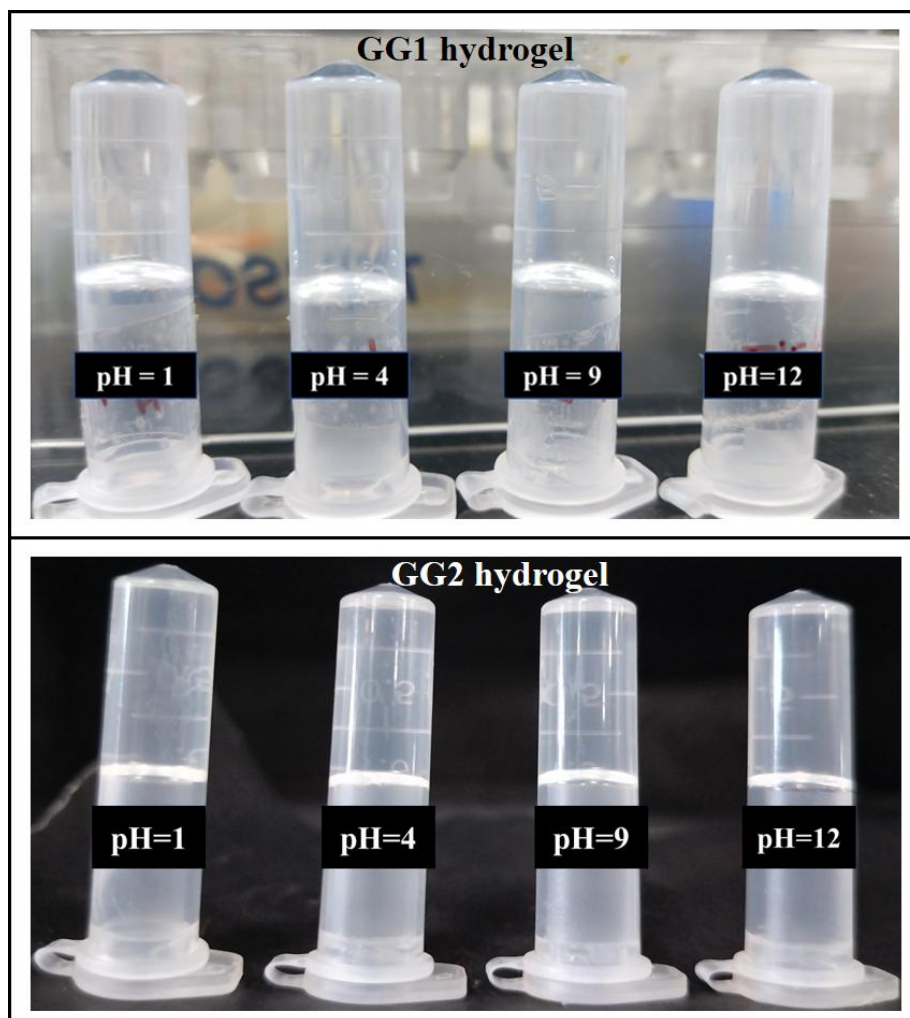


Figure 4.4 Digital images showing the pH stability of both hydrogels.

4.2.2 Morphological Characterization of Self-Assembled Hydrogels

The morphological evaluation of synthesized hydrogels was done using several microscopic techniques. Field emission scanning electron microscopy (FESEM) micrographs of GG1 and GG2 hydrogels revealed the highly entangled fibrillar morphology in both gels. The obtained fibers were helical, as evident from the images (**Figure 4.5a, 4.5b**). The diameter of the individual fiber was calculated as 25-35 nm in the case of GG1 gel and 40-60 nm in the case of GG2 gel, and was

several microns in length in both cases. This indicated the bundling of individual strands together to form the nanofibers.

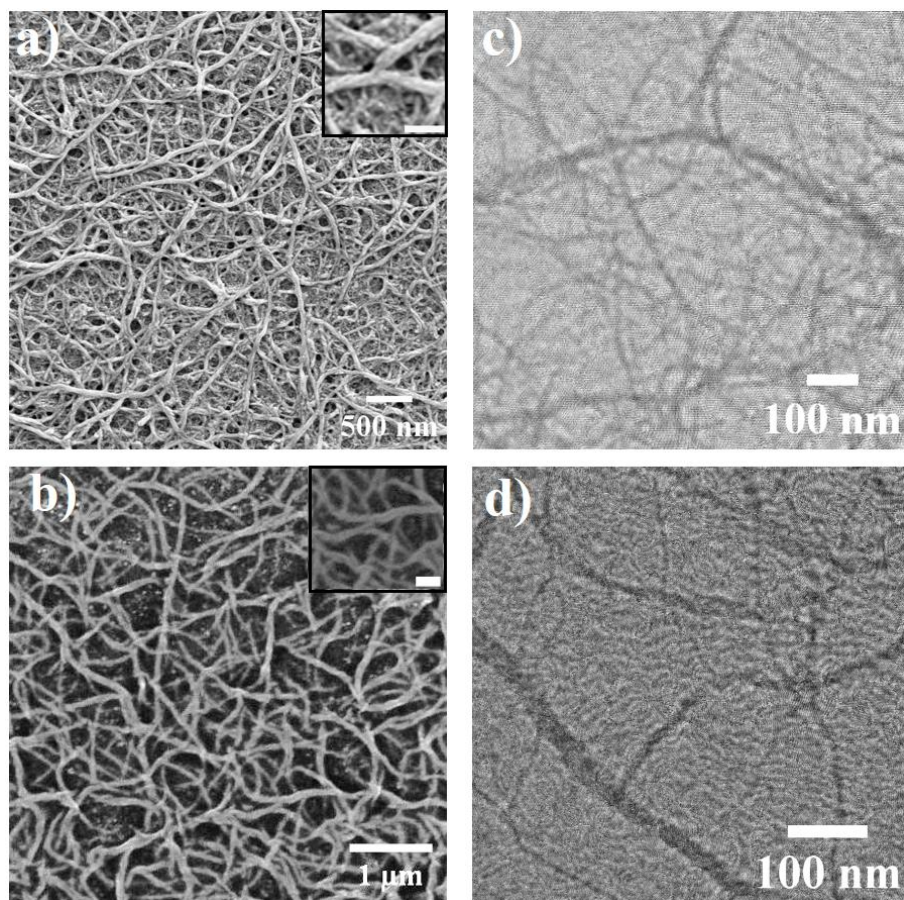


Figure 4.5 a,b) FESEM images of GG1 and GG2 hydrogels, respectively (Inset: Enlarged FESEM image showing helicity, scale bar 200 nm), c,d) TEM images of GG1 and GG2 hydrogels, respectively.

Transmission electron microscopy also supported the formation of intertwined fibrillar morphology in both the gels as obtained from FESEM images. The diameter of the fiber calculated from TEM images was in the range of 17-20 nm for GG1 hydrogel, and 19-22 nm in the case of GG2 hydrogel (**Figure 4.5c, 4.5d**). Atomic force microscopy (AFM) was also employed to investigate the morphological aspects of the hydrogels. The highly entangled, dense network of helical fibers was observed in the AFM images of both the hydrogels (**Figure 4.6a, 4.6b**). The height of the fibers was also calculated and found to be around 5-8 nm.

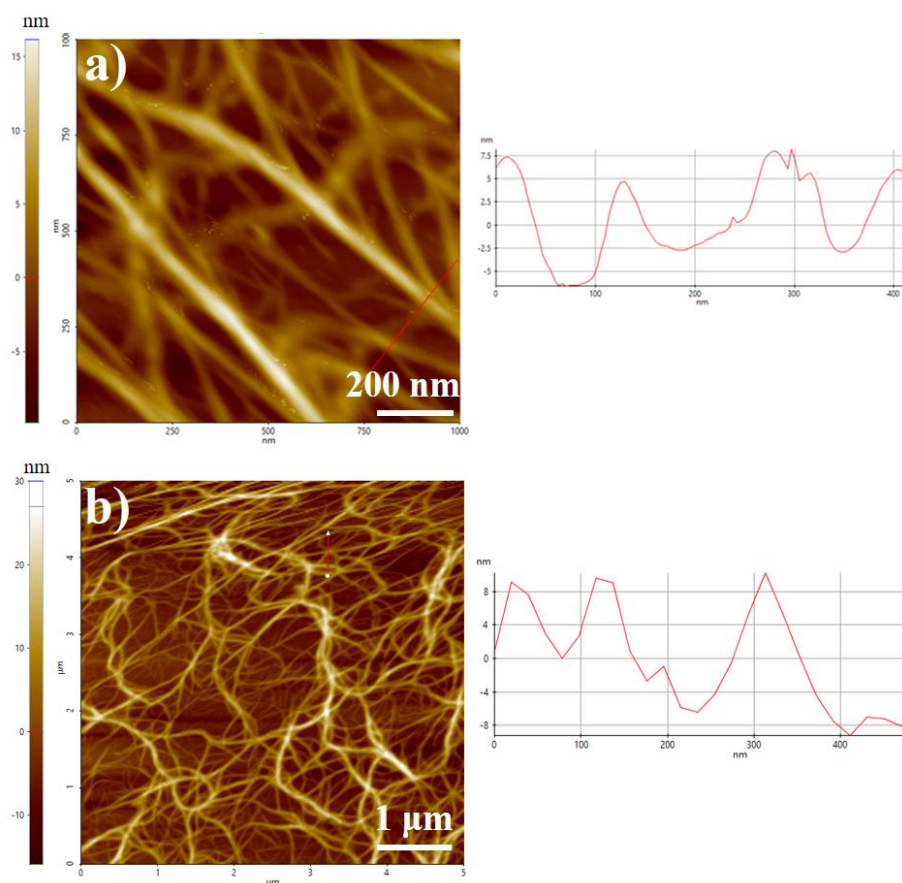


Figure 4.6 a,b) AFM images of GG1 and GG2 hydrogels with their height profiles.

4.2.3 Spectroscopic Characterization of Hydrogels

UV-visible spectrum of GMP showed an absorption maximum at 252 nm along with a shoulder peak at 274 nm. The addition of GSH and GSSG solution to the GMP led to the hypochromic shift characteristic of the formation of an ordered self-assembly in both the gels (**Figure 4.7a, 4.7b**) [35]. The Job's plot was also derived using UV-visible spectroscopy by varying the mole fractions of GSH, GSSG, and GMP, which suggested the optimum interaction takes place at a stoichiometric ratio of 1:1 for GSH: GMP and GSSG: GMP (**Figure 4.7c-4.7f**).

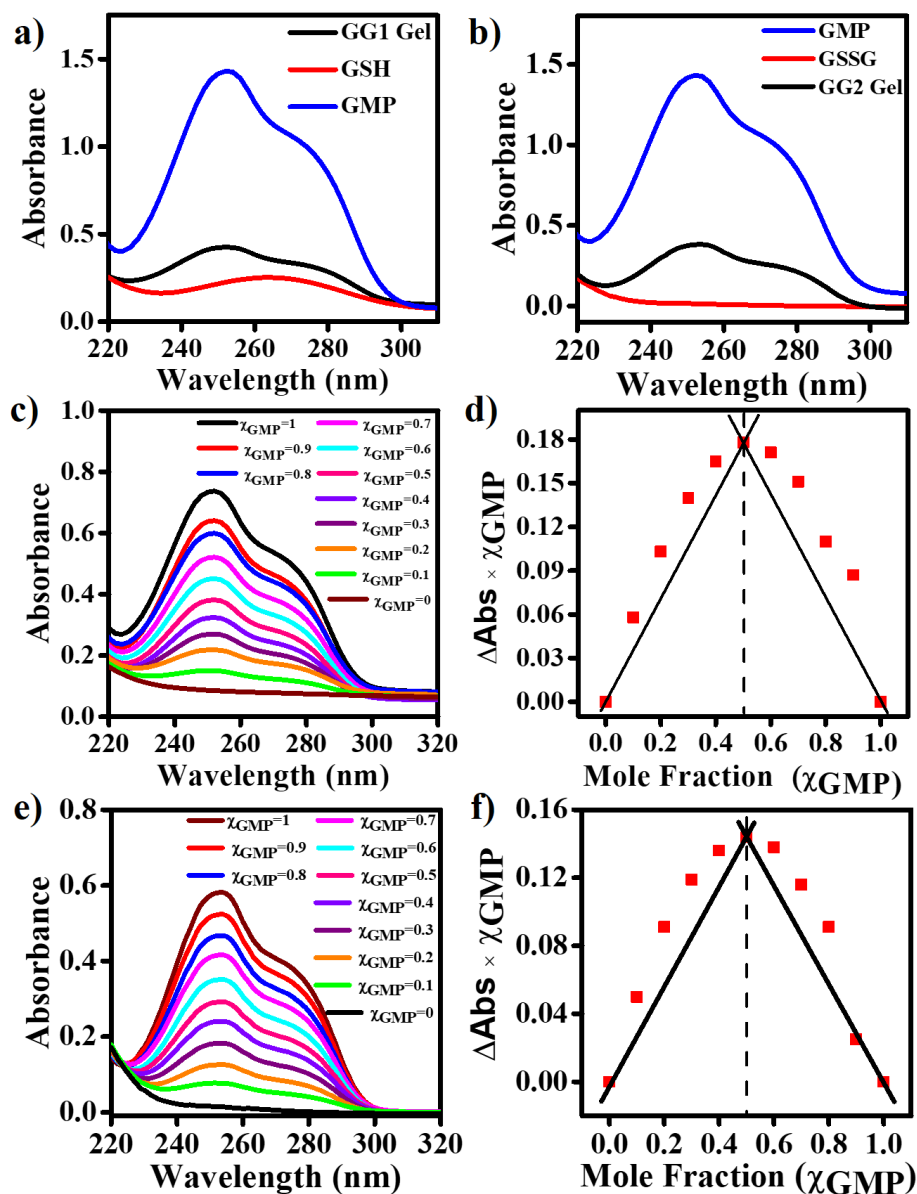


Figure 4.7 a,b) UV-visible spectra of GG1 and GG2 hydrogels, c,e) UV-visible spectra of GMP upon addition of different amounts of GSH and GSSG respectively, d,f) Job's plot obtained at its maximum absorption wavelength at 252 nm.

Guanosine and its derivatives, including GMP, are well known for the formation of higher-order self-assembled G-motifs like dimers, G-quartets, quadruplexes, etc [36]. To further investigate the type of G-motif, we employed thioflavin T (ThT), a fluorogenic dye known to show significant enhancement in fluorescence intensity upon binding with G-quartets. The strong non-covalent interactions experienced by ThT from G-quadruplex upholds the dye in a rigid and planar environment, thus restricting its non-radiative pathways [37,38]. To

observe this, 10 μL of 1 mM ThT solution was added to both GG1 and GG2 hydrogel (1 mL). The emission spectrum was recorded for blank ThT solution and ThT incorporated hydrogels at an excitation wavelength of (λ_{ex}) 445 nm. Blank ThT showed a very low intensity peak at 491 nm, while a very intense fluorescence peak was observed at 491 nm in the case of ThT incorporated hydrogels. The enhancement in fluorescence could also be confirmed visually when the vials were exposed to long-range UV light (**Figure 4.8a and 4.8b inset**). The fluorescence intensity (F_L/F_0) increases by 78 and 85 times for GG1 and GG2 hydrogels, respectively (**Figure 4.8a, 4.8b**).

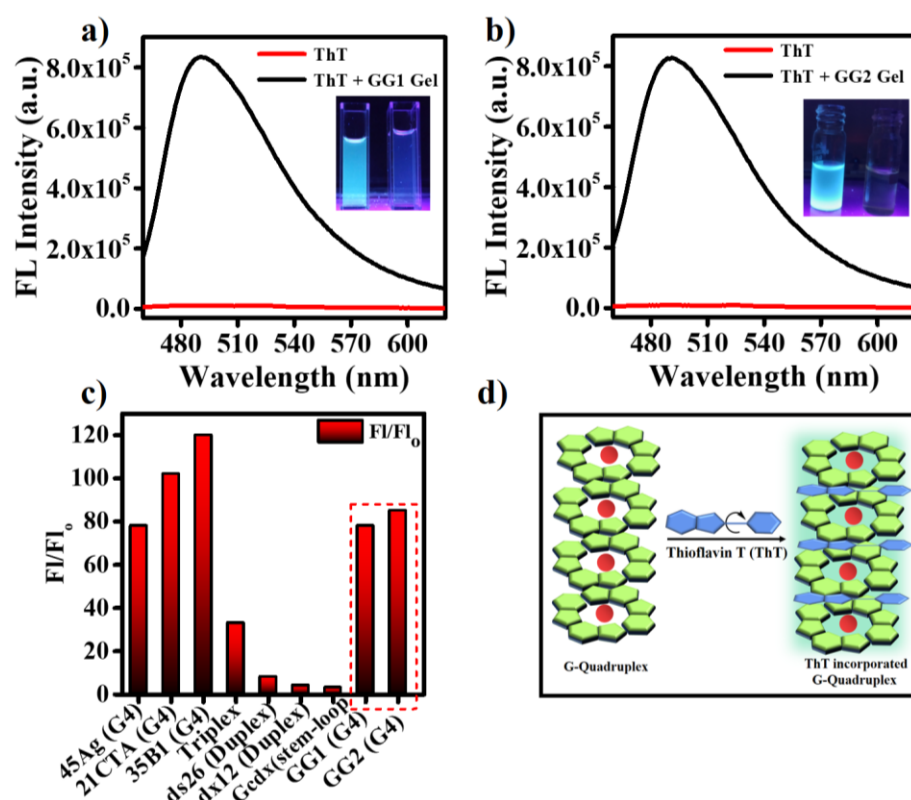


Figure 4.8 a,b) Fluorescence spectra of blank ThT and ThT incorporated GG1 and GG2 hydrogels respectively, c) Comparison of fold enhancement in fluorescence intensity of ThT incorporated hydrogels with other reported assemblies, d) Plausible mechanistic depiction for enhancement in intensity of ThT when incorporated in hydrogel.

This significant enhancement depicts the formation of G-quadruplex, which is in agreement with earlier reported quartet systems (**Figure 4.8c**). This amplification in fluorescence intensity is observed due to the binding of ThT between the π - π stacked fibrils (**Figure 4.8d**). On the contrary, the fluorescence intensity of the ThT incorporated gel was significantly quenched with an increase in temperature (**Figure 4.9a, 4.9c**). At higher temperatures, the ThT molecules that were initially entrapped in a more rigid and planar network are now free to move, thus activating the non-radiative pathways [37]. The reversibility in the fluorescence enhancement and quenching was also observed with respect to temperature, indicating the dynamic self-assembly in the hydrogels (**Figure 4.9b, 4.9d**).

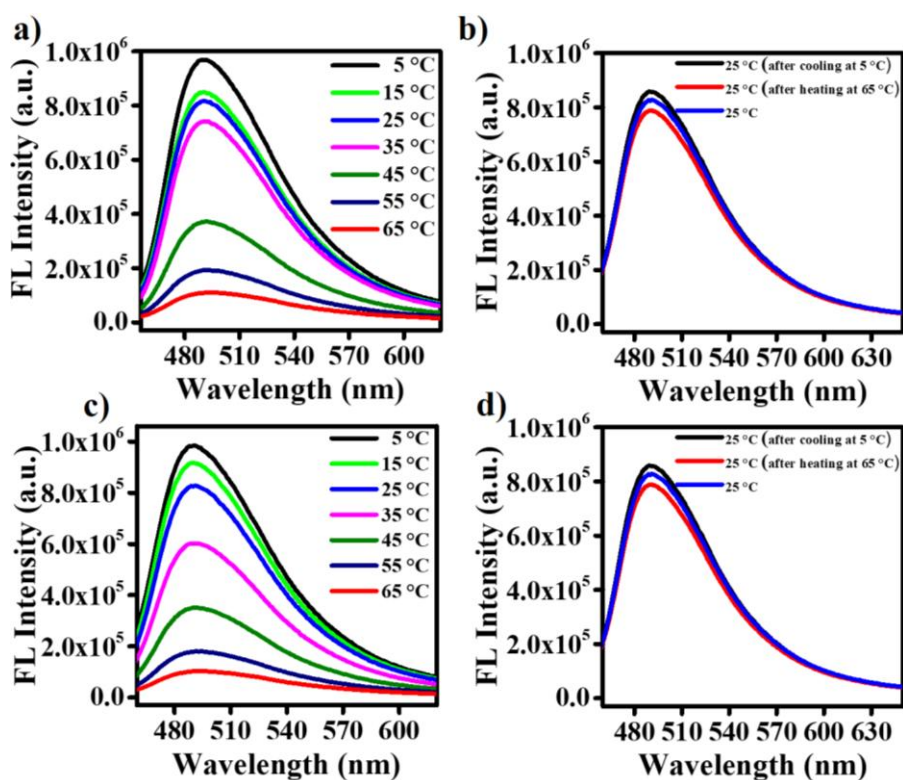


Figure 4.9 a,c) Temperature-dependent fluorescence study of ThT incorporated GG1 and GG2 hydrogels respectively, b,d) Fluorescence spectra depicting the reversibility of fluorescence enhancement and quenching of ThT incorporated GG1 and GG2 hydrogels respectively.

The formation of G-quartet was further supported by wide-angle powder X-ray diffraction (P-XRD). GG1 xerogel exhibited a distinct peak at $2\theta = 4.0^\circ$ ($d = 2.2$ nm) that could be assigned to the diameter of the single G-quartet. Another major peak was observed at $2\theta = 27.35^\circ$ with a d value of 0.33 nm, which signifies the π - π stacking distance between adjacent planar G-quartets. Similar peaks were observed for GG2 hydrogel at $2\theta = 3.9^\circ$ with a d value of 2.3 nm and $2\theta = 27.84^\circ$ with an interplanar distance of 0.32 nm (**Figure 4.10a-4.10c**). These evidences altogether suggested the presence of G-quadruplex in the structure of both the hydrogels [39,40].

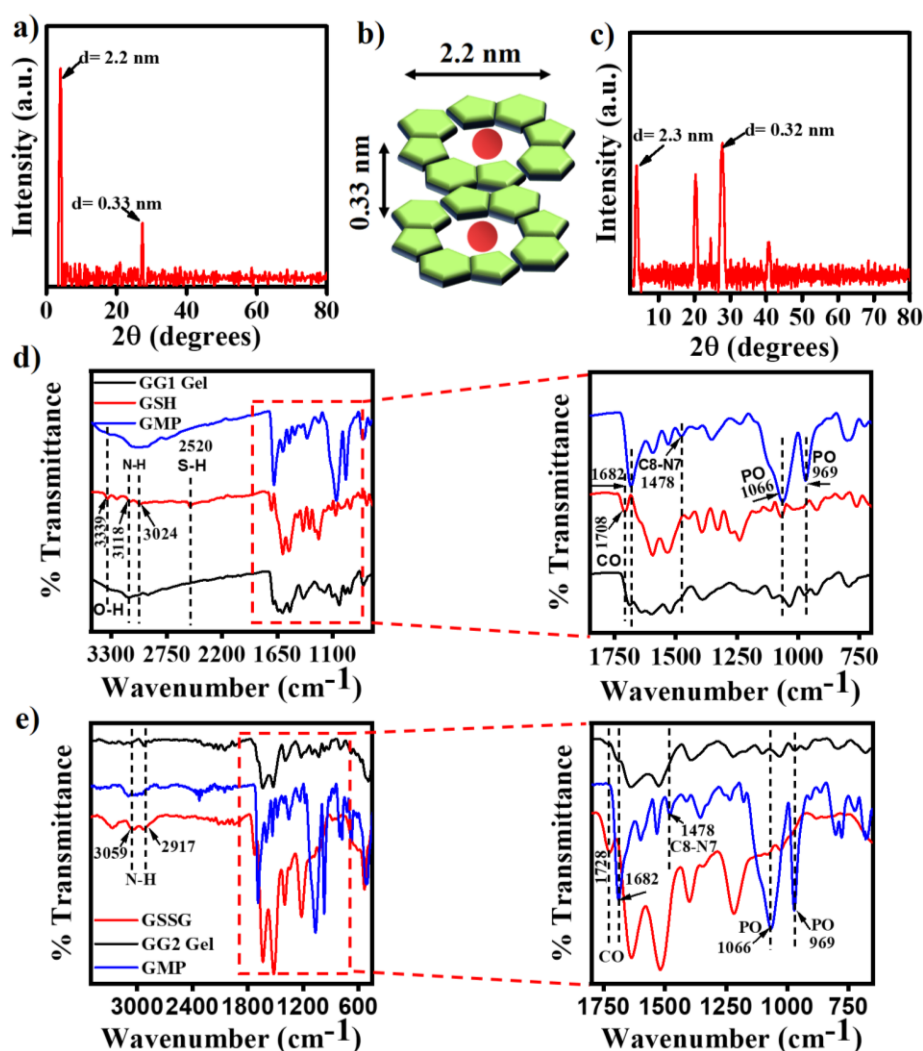


Figure 4.10 a,c) PXR spectra of GG1 and GG2 hydrogels, respectively, b) Graphical representation showing the dimension of G-quartet and their interlayer separation, d,e) FTIR spectra of GG1 and GG2 hydrogels, respectively.

FTIR spectra of freeze-dried hydrogels were recorded to investigate the molecular interactions and the functional groups responsible for gel formation (**Figure 4.10d, 4.10e**). The peaks at 3118 and 3024 cm^{-1} in GSH, corresponding to N-H stretching frequency, shifted to 3141 and 3054 cm^{-1} , respectively, in GG1 gel. Similarly, in the case of GSSG, the peaks at 3059 and 2917 cm^{-1} shifted to 3081 and 2932 cm^{-1} in the GG2 gel, indicating the interaction of the NH_2 group of GSH and GSSG with the GMP in the hydrogel matrix. The peaks at 1708 cm^{-1} for GSH, 1728 cm^{-1} for GSSG, and 1682 cm^{-1} for GMP, corresponding to carbonyl stretching frequency, shifted to 1690 and 1691 cm^{-1} in GG1 and GG2 gels, respectively, confirming the involvement of the C=O group in the gel formation. The peak at 2520 cm^{-1} in GSH disappeared in GG1 hydrogel, indicating the involvement of the thiol group in H-bonding. Further, the peak at 1478 cm^{-1} in GMP corresponding to C8-N7 stretching frequency disappeared in both GG1 and GG2 gels, confirming the intermolecular-hydrogen-bonded cyclic arrangement in the G-quartet. Two peaks at 1066 and 969 cm^{-1} are assigned to the asymmetric and symmetric stretching frequencies of the phosphate group in GMP. These peaks displayed a considerable shift to 1103 and 1037 cm^{-1} in GG1 gel and 1101 and 1035 cm^{-1} in GG2 gel, respectively. This indicates the involvement of the phosphate group in the gel matrix [41,42]. The phosphate group interaction was further validated by observing an appreciable upfield shift in ^{31}P NMR spectra (**Figure 4.11**). The peak at 3.47 ppm in GMP shifted to 0.19 ppm and 0.57 ppm in GG1 and GG2 hydrogels, respectively, depicting the involvement of the phosphate group in gelation. The NH_2 group of GMP also plays an important role in G-quartet formation. The gelation was not observed when IMP was used instead of GMP as a gelator for interaction with GSH or GSSG. It is structurally similar to GMP but lacks the NH_2 group. This observation also supported the formation of the G-quartet and its crucial role in gelation. The plausible binding of carbonyl and amine groups of GSH and GSSG occurred preferably with the amine and phosphate group of GMP, respectively.

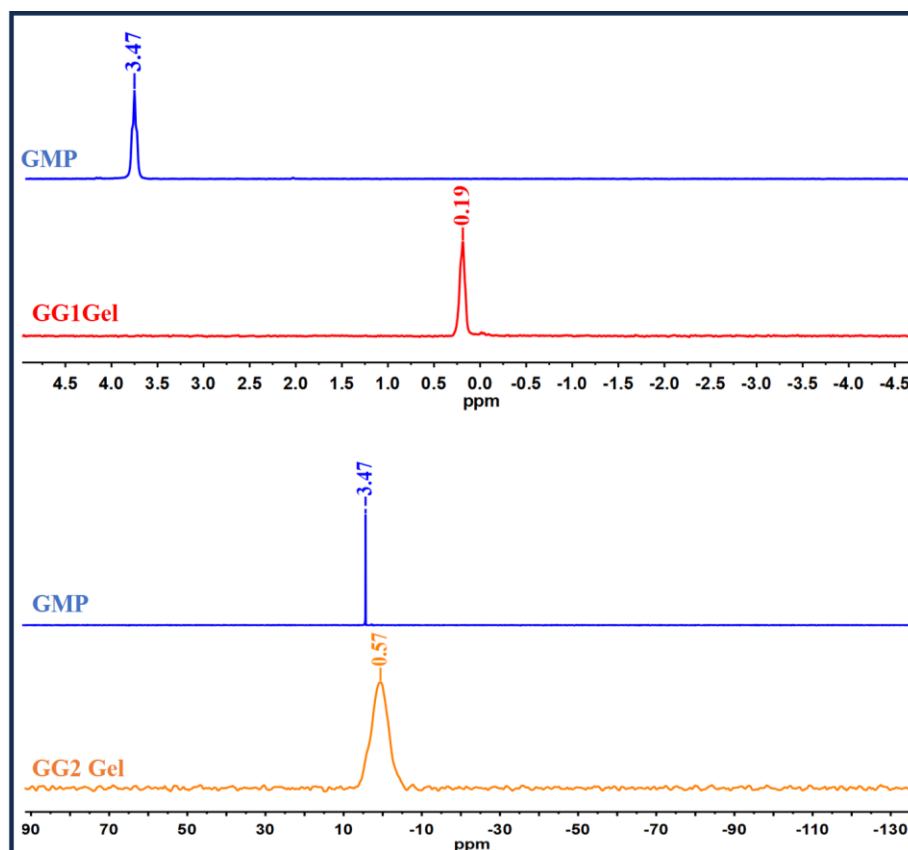


Figure 4.11 ^{31}P NMR spectra of GG1 and GG2 hydrogels.

Based on several spectroscopic and microscopic characterizations, a plausible structure for GG1 and GG2 hydrogels is proposed (**Figure 4.12**). The FTIR and ^{31}P NMR suggested the plausible interaction of phosphate and amine group in GMP with the amine and carbonyl group of GSH or GSSG, respectively. Further, the G-quartet formation in the hydrogels was confirmed by PXRD and ThT binding assay. The stacking of these G-tetrads was facilitated via π - π stacking of the aromatic moiety. Several other non-covalent interactions, like hydrophobic interactions, electrostatic interactions, along with π - π stacking, resulted in the formation of self-assembled GG1 and GG2 hydrogels.

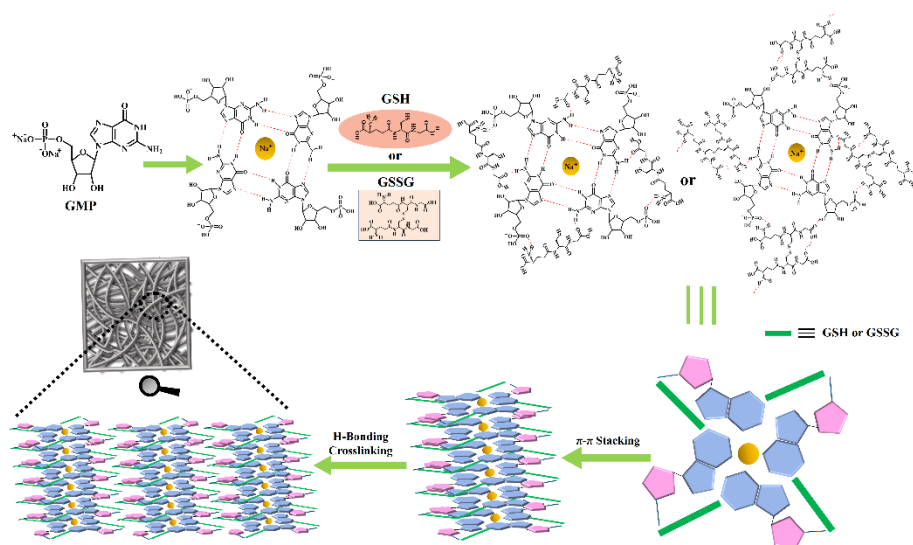


Figure 4.12 A schematic representation for the plausible G-Quartet formation leading to hydrogel formation.

4.2.4 Rheological Properties of Hydrogels

The mechanical properties and viscoelastic behaviour of the hydrogels were investigated by rheological studies. The amplitude-sweep experiments revealed that the value of storage modulus (G') is greater than the value of loss modulus (G'') indicating the dominance of elastic property over the viscous property up to a strain value of 145% and 147% for GG1 and GG2 hydrogels respectively (**Figure 4.13a, 4.13b**). Further, the frequency sweep experiment at a constant strain value also confirmed the dominance of G' over G'' (**Figure 4.13c, 4.13d**). The thixotropic loop test was performed to understand the robustness and strain-bearing capacity of the hydrogel (**Figure 4.13e, 4.13f**). The alternate low and high strains of 1% and 500% were applied for a definite interval of time period for 5 consecutive cycles for both the hydrogels. At a strain of 1%, the gels were elastic ($G' > G''$). On the other hand, as the strain was increased to 500%, the gels started losing their elastic behaviour ($G' < G''$). The release of strain again led to the dominance of elastic behaviour over the viscous property within a very short interval of time, indicating that both GG1 and GG2 hydrogels were thixotropic in nature. Since injectable property and thixotropic

behaviour are often interlinked, these hydrogels can be utilized as a soft injectable material.

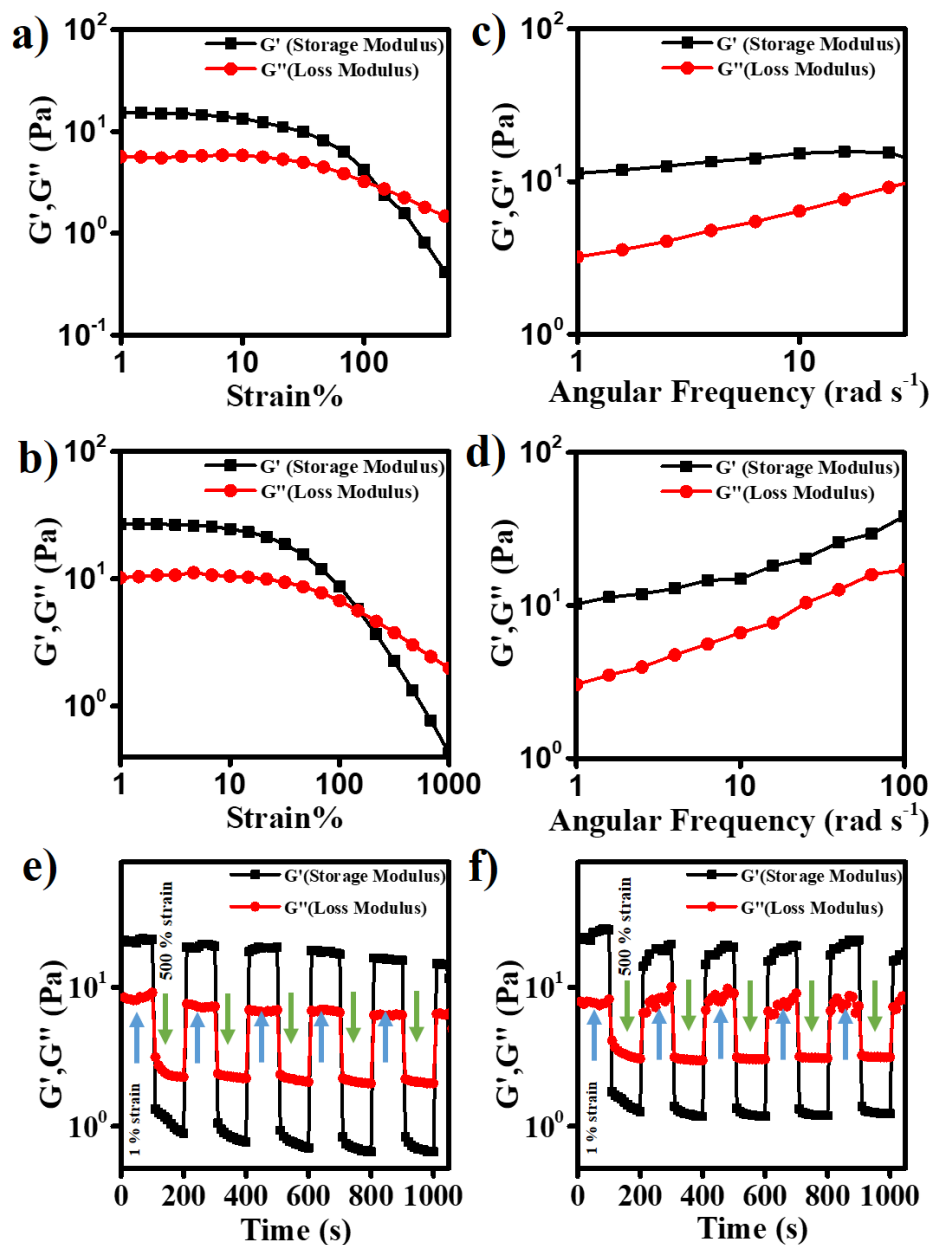


Figure 4.13 a,b) Dynamic Strain-Sweep studies for GG1 and GG2 hydrogels. c,d) Frequency- Sweep measurement at a constant strain of 5%. e,f) Thixotropic loop test for GG1 and GG2 hydrogels at an alternative strain of 1% and 500%.

4.2.5 Morphological Evolution of Nucleotide-Governed Peptide Self-assembly to Hydrogel Formation

Peptide-nucleotide self-assembly involves the spontaneous organization of these molecules into higher-order structures owing to several non-covalent interactions [43,44]. It is a crucial phenomenon as natural peptide-nucleotide complexes like ribosomes inspire synthetic assemblies [45].

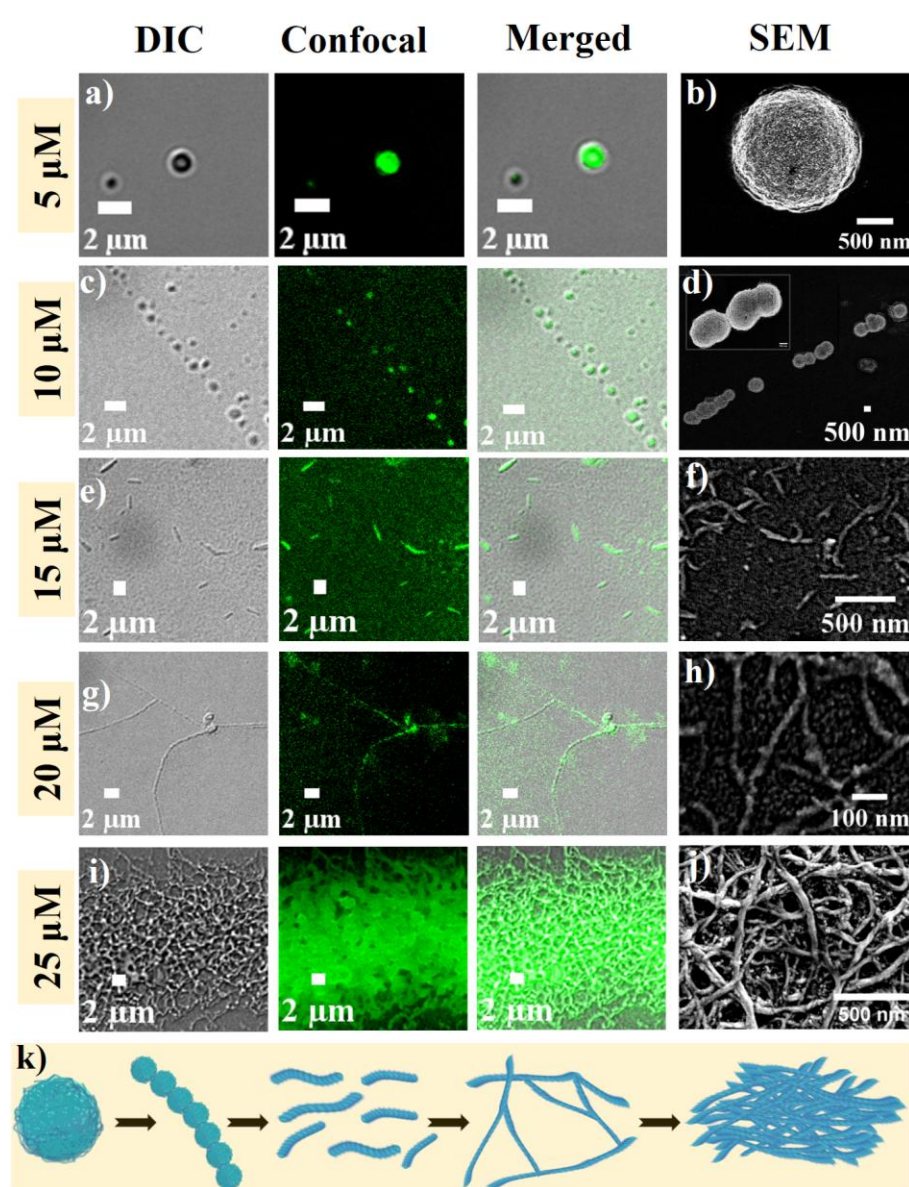


Figure 4.14 . a-j) Confocal and FESEM images depicting morphological evolution at constant GSH concentration (100 μM) while varying the concentration of GMP using ThT. k) Schematic representation of the self-assembly during hydrogel formation.

Moreover, their co-assembly might play an important role in the emergence of protocells. To gain more insight into the self-assembly process during the hydrogel formation of GSH and GMP, concentration-dependent kinetic studies were carried out using several microscopic techniques. Since GMP plays a prominent role in the formation of stacked fibrils and quartet assembly, we investigated the morphological evolution at variable concentrations of GMP while keeping the concentration of GSH fixed (100 μM). At a very low concentration of GMP (5 μM), the random peptide molecules organized into spherical aggregates (**Figure 4.14a, 4.14b**). A further increase in GMP concentration to 10 μM , resulted in more spherical aggregates that started to fuse and assembled in an elongated linear fashion resulting in the formation of protofibrils (**Figure 4.14c, 4.14d**). At an increased concentration of 15 μM of GMP, these protofibrils undergo fragmentation to form smaller nanofibers (**Figure 4.14e, 4.14f**). At 20 μM GMP, hydrophobic interactions and other non-covalent interactions promote stacking of these nanofibers to form elongated fibers with prominent helicity (**Figure 4.14g, 4.14h**) [46]. These fibers intertwined together to form a highly entangled fibrillar network at a higher concentration of GMP (30 μM) (**Figure 4.14i, 4.14j**) [47]. The morphological evolution of spherical aggregates to stacked entangled fibrillar formation is depicted in **Figure 4.14k**.

4.2.6 Cell Cytotoxicity Analysis

Cell viability tests for GG1 and GG2 were carried out in concentrations ranging from 0 to 200 μM in gastric epithelial cells. As indicated in **Figure 4.15**, there was a steady decline in cell viability with an increase in concentrations of the hydrogels. The half maximal inhibitory concentration IC_{50} values for GG1 and GG2 were 714 and 902 μM , respectively. The IC_{10} values for GG1 and GG2, 29 and 84 μM , were used, respectively, for further experimentation. Such a value indicates minimal toxicity in eukaryotic cells while still carrying potential selectivity against bacteria, as discussed in further sections.

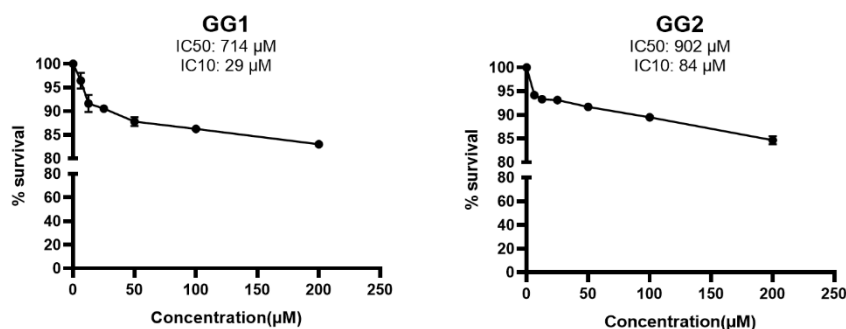


Figure 4.15 Cell cytotoxicity analysis of GG1 and GG2 compounds on gastric epithelial cells (AGS) using MTT assay. Upon 24 h of incubation, the compounds showed an IC₅₀ of 714 and 902 µM for GG1 and GG2, respectively.

4.2.7 Potent Antiviral Activity of Hydrogels upon EBV Infection and SARS-CoV-2 Spike Transfection

To further evaluate the antiviral activity of these hydrogels, we infected AGS cells with Epstein-Barr virus, a double-stranded DNA virus, and transfected A549 cells with SARS-CoV-2 (double-stranded RNA virus) spike variant of concern. EBNA1 was significantly upregulated ($p < 0.01$) in EBV-exposed AGS cells. A significant downregulation of *EBNA1* transcripts ($p < 0.01$) was shown upon hydrogel treatments. EBNA1 is required for replication of the virus and activates the expression of viral latent genes. In addition to maintaining viral latency, EBNA1 also promotes cell proliferation in EBV-associated cancers. Suppression of EBNA1 expression upon hydrogel treatment indicated the potential of these compounds in inhibiting viral persistence and replication (**Figure 4.16a**). Moreover, SARS-CoV-2 Spike protein-transfected A549 cells indicated reduced expression of Spike at transcript levels ($p < 0.01$) in both GG1 and GG2-treated cells (**Figure 4.16b**). Spike protein provides a crucial anchorage to bind to host cells and induce viral RNA expression and thereby promote SARS-CoV-2-mediated pathogenesis. A reduction in Spike transcripts could possibly indicate a lower binding affinity of SARS-CoV-2 on host cells, thus showing antiviral activity against SARS-CoV-2. This suggested that

hydrogel treatment was efficient against both DNA and RNA virus infections, further supporting their potential antiviral activities.

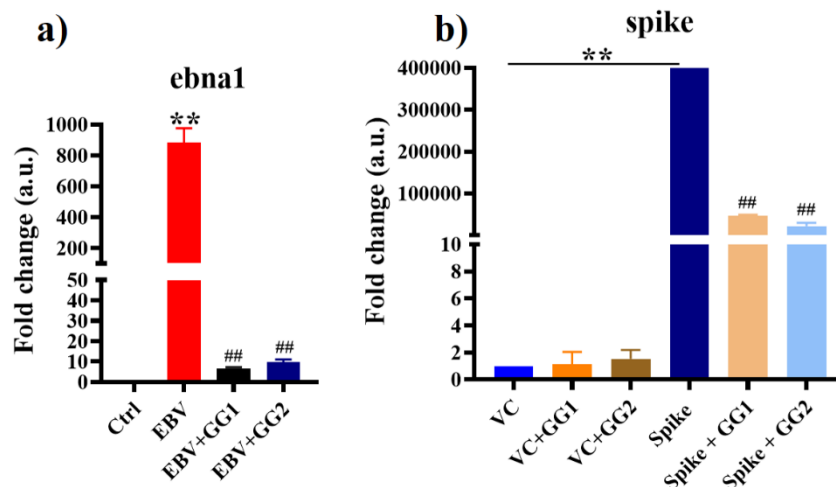


Figure 4.16 Hydrogel treatment reduced the viral load. a) Bar graph represents the transcript expression of EBNA1 gene; b) Bar graph representing a significant reduction in Spike transcript levels as observed in GG1 and GG2 treated lung epithelial cells. Unpaired t-tests were applied to determine the significance wherein p -values < 0.05 , 0.01 , and 0.001 were considered statistically significant with */# denoting upregulation/downregulation respectively.

4.2.8 Antibacterial Activity of Hydrogels against Gram-Positive and Gram-Negative Bacteria

The antibacterial effect of both the hydrogels carries enormous potential in the development of a wide variety of biomedical applications. Therefore, we evaluated the in vitro antibacterial activities of GG1 and GG2 hydrogels against both gram-negative (*E. coli*) and gram-positive (*B. subtilis*) bacteria. The hydrogels were challenged with both pathogens at a concentration of 10^6 CFU/mL, and the bacterial colonies were counted after 12-16 h incubation. When compared to negative control, GG1 was able to significantly reduce survival by 83% ($p < 0.001$) in *E. coli* (Figure 4.17a, 4.17b). On the other hand, GG2 hydrogel was able to significantly reduce bacterial survival by 56% in *E. coli* ($p < 0.05$) and by 71% in *B. subtilis* ($p < 0.05$) (Figure 4.17a, 4.17c). A physiologically relevant concentration of 0.5 mM thus demonstrated higher bactericidal activity, suggesting potent broad-spectrum antibacterial activity of GG1 and GG2 hydrogels. Earlier

studies have also showed that GSH (0.5 mg/mL) could inhibit *S. typhi* growth [48].

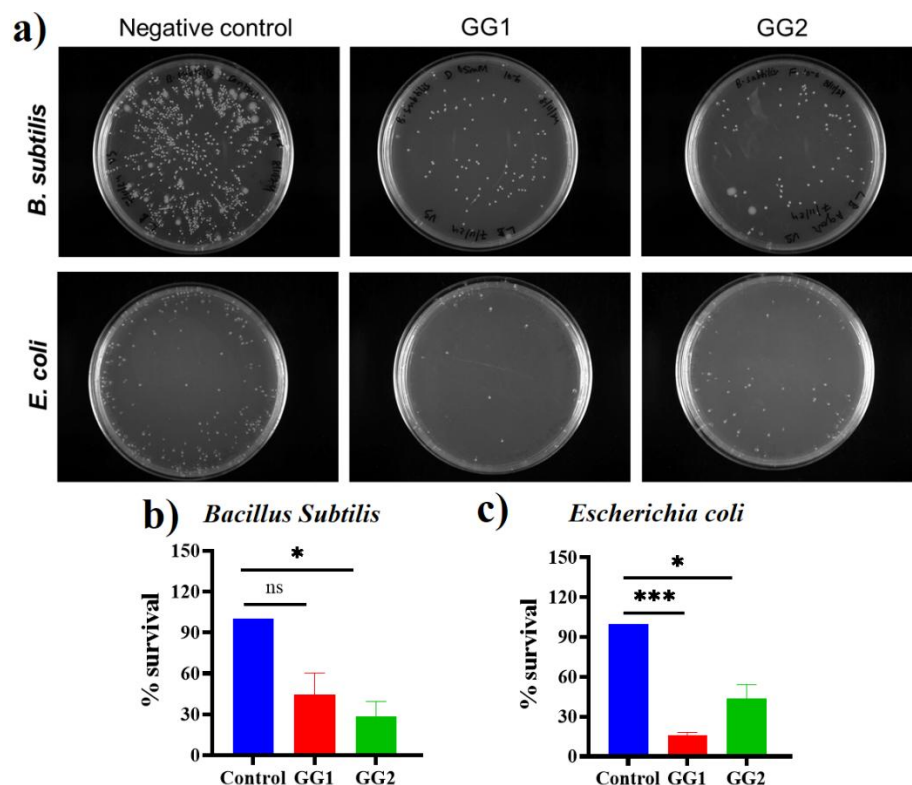


Figure 4.17 a) Representative images of Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria upon exposure to hydrogels, b,c) Quantitative representation of bacterial survival. Unpaired t-tests were applied to determine the significance, wherein p-values < 0.05, 0.01, and 0.001 were considered statistically significant, with */# denoting upregulation/downregulation, respectively.

4.2.9 Hydrogels indicate potent anticancer activity in gastric epithelial cells

The benzopyrene (BaP) induced pro-cancerous conditions in AGS cells, mimicking oncogenicity, is indicated by relevant markers at transcript levels. To assess the potent anticancer activity of hydrogels, cells were treated with hydrogels post-benzopyrene exposure. PCNA is a cell proliferation marker, which was significantly upregulated in BaP-treated cells ($p < 0.001$). PCNA was significantly downregulated ($p < 0.001$ and 0.01) in both GG1 and GG2 treated cells, suggesting that hydrogels reduced the cell proliferation in BaP-induced AGS cells. We did not find much changes in pRb expression in BaP and hydrogels-

treated cells. An inhibitor of apoptosis, *SURVIVIN*, was observed to be upregulated in BaP-treated cells. While the significant downregulation ($p < 0.05$) was seen in GG2-treated cells, indicating inhibition of cell survival pathways. Moreover, various cell cycle markers (*CCND1*, *CDK1*) were significantly upregulated in the case of BaP-treated cells. The downregulation ($p < 0.01$) of cell cycle checkpoint marker, *CCND1*, involved in G1/S transition, in both GG1 and GG2-treated cells, indicates cell cycle arrest, possibly halting oncogenic cell growth. Similarly, checkpoint markers for G2/M transition, *CCNB1*, and *CDK1* were significantly downregulated ($p < 0.01$) in both the treated cells, indicating loss of oncogenic cells to thrive in a cancerous microenvironment. A key player of intrinsic apoptosis, *APAF1*, was downregulated in BaP-treated cells, whereas significant upregulation ($p < 0.001$) was observed in GG1 and GG2-treated cells, indicating cell death progression through the formation of apoptosome and activation of caspases (**Figure 4.18**).

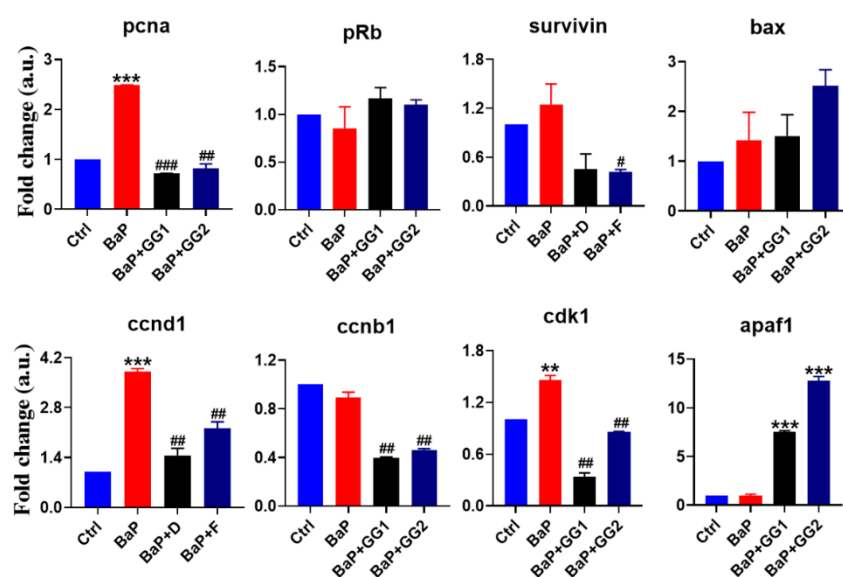


Figure 4.18 Gastric epithelial cells treated with GG1 and GG2: Bar graph represents the transcript analysis of different genes through qRT-PCR, resulting in induced cell apoptosis. Unpaired t-tests were applied to determine the significance, wherein p-values < 0.05 , 0.01 , and 0.001 were considered statistically significant, with */# denoting upregulation/downregulation, respectively.

Furthermore, the efficacy of hydrogels on cell proliferation was also assessed through crystal violet staining. BaP treatment significantly

enhanced the cell proliferation rate in AGS cells ($p < 0.001$). However, the hydrogel treatment indicates a significant reduction in cell proliferation (p -values < 0.01) when compared to benzopyrene-exposure alone (**Figure 4.19**). This suggested the role of hydrogels in targeting key pathways by inhibiting cell proliferation and activation of programmed cell death.

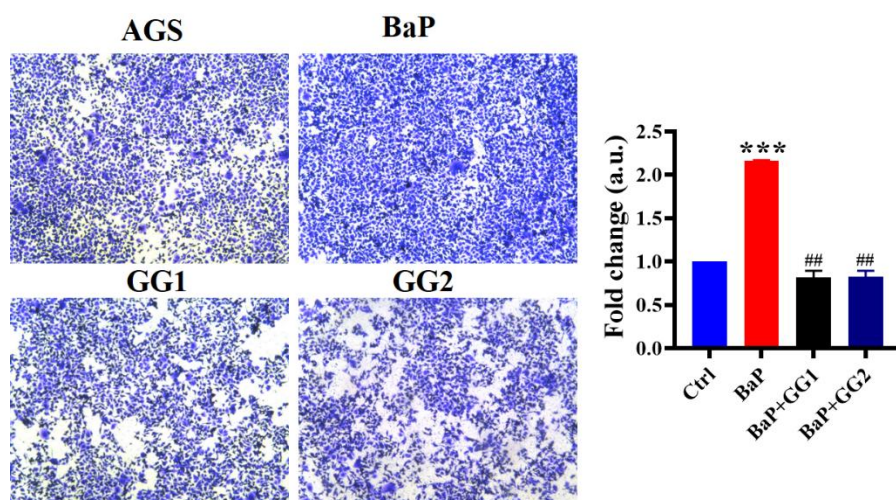


Figure 4.19 Gastric epithelial cells treated with GG1 and GG2 display enhanced cell death and reduced cell proliferation: Crystal violet staining images indicate reduced cell proliferation upon hydrogels treatment. Unpaired t -tests were applied to determine the significance, wherein p -values < 0.05 , 0.01 , and 0.001 were considered statistically significant, with */# denoting upregulation/downregulation, respectively.

4.2.10 Hydrogels treatment induces apoptosis in gastric milieu

Programmed cell death is a key event suggesting the activation of apoptotic pathways to reduce cancer progression. EB/AO live cell staining method is a remarkable technique to indicate apoptotic, necrotic, and viable cells, indicated by orange/yellow, red, and green stained cells, respectively. The percentage of live cells was reduced in GG1 and GG2-treated cells as compared to BaP-exposed cells alone. A significant increase in apoptotic cells ($p < 0.01$) was observed in GG2-treated AGS cells as compared to BaP exposure alone, suggesting that hydrogels are inducing cancer cell death. (**Figure 4.20a, 4.20b**).

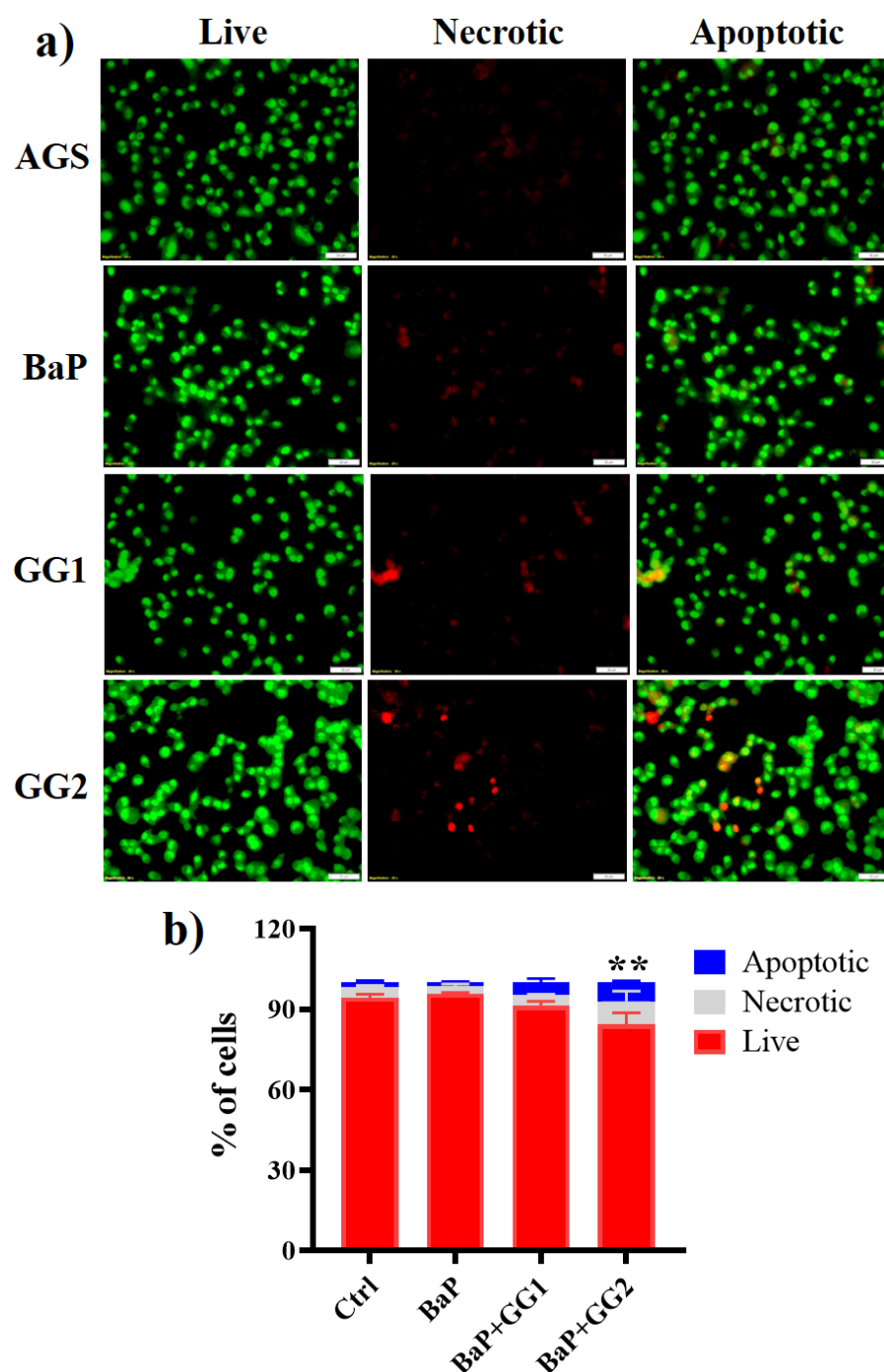


Figure 4.20 Hydrogel treatment post BaP exposure induces apoptosis in gastric cells.

a) Representative images of EB/AO stained AGS cells show the different apoptotic phases after hydrogel treatment; b) Quantitative representation of EB/AO stained AGS cells. The experiment was performed in triplicate with a total of 1000 cells counted for each set to determine live, apoptotic, and dead cells. Unpaired t-tests were applied to determine the significance, wherein p -values <0.05 , 0.01 , and 0.001 were considered statistically significant, with */# denoting upregulation/downregulation, respectively.

4.3 Conclusion

In summary, supramolecular hydrogels were fabricated through a simple mixing of reduced glutathione or oxidized glutathione with GMP in aqueous medium under ambient conditions. The fibrillar nanostructures in the hydrogels were obtained due to the formation of a G-quadruplex matrix, followed by π - π stacking and extensive hydrogen bonding. The hydrogels were stable at room temperature and showed thermo-reversible, shape-sustainable, self-healing, and thixotropic behaviour. Both the hydrogels were stable in a wide range of pH window from 1 to 12 and were mechanically robust. This simple synthetic protocol involves no chemical modification of the precursor peptide biomolecules, thereby preserving their pristine form in the hydrogels. The growth mechanism for fiber formation was investigated by concentration-dependent confocal studies. Initial protocell-like spherical intermediates gradually transformed to helical fibers via the formation of protofibrils with an increase in the nucleotide concentration. The gels were highly biocompatible. In vitro cell studies revealed the inherent antiviral activities of the hydrogels against EBV and SARS-CoV-2 viruses. Both the hydrogels also showed antibacterial activity against both gram-positive and gram-negative bacteria at a physiologically relevant concentration of 0.5 mM. The anticancer activity of the hydrogels was also explored, and qRT-PCR revealed the induced cell apoptosis upon treatment with hydrogels. EB/AO staining also suggested the potential anticancer activity of the hydrogels. Thus, the developed peptide-nucleotide hydrogels have enormous potential to be utilized as multimodal therapeutic agents. The ease of synthesis, inherent potential of the hydrogel for a variety of biological applications, and potential of hydrogels to be easily integrated onto various surfaces make the system easily applicable for the prevention of infections and wound healing.

4.4 Experimental Section

4.4.1 Materials

Reduced Glutathione (GSH), Oxidized glutathione (GSSG), Guanosine Monophosphate disodium salt (GMP- Na_2), Adenosine 5'-monophosphate disodium salt (AMP- Na_2), Cytosine 5'-monophosphate (CMP), Inosine 5'-monophosphate disodium salt dihydrate (IMP- Na_2), Thioflavin T (ThT), Rhodamine B (RhB), Methylene Blue (MB), Water HPLC grade, were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL- India). Gastric (AGS) and lung (A549) epithelial cells were procured from the National Centre for Cell Science (NCCS, Pune, India). Plasmids containing the SARS-CoV-2 Spike gene, wildtype (pCMV3-C-GFPSpark SARS-CoV-2 2019-nCoV, Cat. no. VG40589-ACG) and vector control (pCMV3-C-GFPSpark, Cat. no. CV026) were purchased from Sino Biologicals (Sino Biological, Beijing, China). Roche X-treme GENE HP DNA Transfection Reagent was purchased from Merck, Darmstadt, Germany (Cat. no. 06366236001). TRIzol reagent (Sigma, Life Sciences, Sigma-Aldrich, St. Louis, USA). cDNA synthesis was performed using a synthesis kit procured from PrimeScript RT Master Mix; Takara, Shiga, Japan. SYBR green master mix was obtained from Agilent Technologies, Santa Clara, USA, and Luria Bertini agar was purchased from LB, Becton, Dickinson and Company, New Jersey, USA.

4.4.2 Instrumentation

UV-visible studies were performed using a Perkin-Elmer Lambda 750 spectrophotometer at room temperature. The microscopic images of the hydrogel were obtained using a Nova Nano FE-SEM instrument after coating the dropped cast hydrogel sample with Au. TEM (Transmission Electron Microscopy) measurements were performed using a 300 kV FEI Tecnai G2, F30 instrument. AFM experiments were carried out using an NX-10 PARK system in a tapping

mode, by depositing the diluted hydrogel on mica. The powder X-ray diffraction pattern for freeze-dried gel was recorded on a Rigaku Smartlab instrument having a Cu K α source with 0.154 nm X-ray wavelength. The fluorescence studies were performed using the Fluoromax-4p spectrofluorometer from Horiba Jobin Yvon (FM-100). Fluorescence spectra were analyzed using OriginPro 2016 64 Bit software. FTIR studies were performed using a Bruker ALPHA 11 spectrometer. MHz AVANCE NEO Ascend 500 Bruker BioSpin, a 500 MHz spectrometer was used to record ^{31}P Nuclear Magnetic Resonance (NMR) spectra. Rheological measurements of the fabricated hydrogels were recorded on an Anton Paar Physica MCR 301 rheometer at room temperature. The dynamic strain sweep measurements were carried out at a constant angular frequency of 10 rad s $^{-1}$ and frequency sweep experiments were measured at a 5% constant strain value. The thixotropic loop test studies were performed by changing the strain value from 1 to 500% at a constant 10 rad s $^{-1}$ of angular frequency for every time interval.

4.4.3 Synthesis of Hydrogels

0.2 M stock solutions of reduced glutathione (GSH), oxidized glutathione (GSSG), and guanosine monophosphate (GMP) were prepared by dissolving them in Milli-Q water. Both GSH and GSSG were separately mixed with GMP in an equimolar ratio of 1:1 to obtain a resultant concentration of 100 mM. Both the mixtures were slightly heated to 50 °C. Hydrogels were obtained spontaneously upon cooling to room temperature. Gelation was visually confirmed by the vial inversion test, where no downward flow of the solvent was observed under the influence of gravity, thus confirming the gelation in both cases.

4.4.4 Thioflavin T (ThT) Assay

A stock solution of ThT (1 mM) was prepared in ethanol. 10 μL of 1 mM ThT solution was added to the hydrogels (1 mL) and incubated for 24 hours. The fluorescence was recorded for the blank ThT solution,

ThT incorporated GSH-GMP (GG1), and GSSG-GMP (GG2) hydrogels by exciting at 445 nm, and monitoring the emission at 491 nm.

4.4.5 Mammalian Cell Culture

Gastric (AGS) and lung (A549) epithelial cells were purchased from the National Centre for Cell Science. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM). The media was supplemented with 10% fetal bovine serum (FBS) in addition to penicillin-streptomycin (50 U/mL and 100 µg/mL), and L-glutamine (2 mM) and incubated at 5% CO₂ and 37 °C under humidified conditions [49].

4.4.6 Plasmid isolation and transfection

Plasmids containing the SARS-CoV-2 Spike gene, wildtype (pCMV3-C-GFPSpark SARS-CoV-2 2019-nCoV), and vector control (pCMV3-C-GFPSpark) were used. The plasmids were transformed in *E. coli* DH5α as per the manufacturer's protocol, and concentrations were measured using Nanodrop (Thermo Scientific, Waltham, USA). Further, the transfection of 3 µg of plasmid DNA was performed at 70% confluence using Roche X-treme GENE HP DNA Transfection Reagent. Cells were incubated for 48 h prior to sample processing.

4.4.7 EBV isolation and infection

Genetically engineered EBV-GFP inserted into a bacterial artificial chromosome (BAC) was isolated from stably transfected HEK293T cells grown in complete DMEM with puromycin as a selective antibiotic. The isolation of EBV particles was performed using the previously illustrated protocol [50].

4.4.8 Cell cytotoxicity analysis

For analysing cytotoxicity of GG1 and GG2 compounds, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was performed by seeding A549 and AGS cells at a seeding density of

10,000 cells per well in a 96-well culture plate [51]. The cells were subjected to GG1 and GG2 treatment ranging from 0-100 μ M. After incubation for 24 h, the media was removed, and 0.5 mg/mL MTT reagent was added and incubated for 3 h at 37°C in the dark. Further, dimethyl sulfoxide (DMSO) was added to each well and incubated on an orbital shaker at 220 rpm for 2 h at room temperature. The absorbance at 570 and 590 nm was used to identify the percent viability of cells, considering untreated cells to be 100% viable. The IC₅₀ values were plotted using GraphPad Prism.

4.4.9 Treatment methodology

Each well of a 6-well plate was seeded containing 0.25 million AGS cells. Briefly, in model 1, AGS cells were untreated, while in model 2, AGS cells were treated with BaP and incubated for 48 h. In models 3 and 4, the AGS cells were treated with BaP for 24 h, followed by treatment with GG1 and GG2, respectively, for another 24 h. Additionally, A549 cells were seeded in 0.25 million cell count in each well of a 6-well plate. To check the antiviral activity against RNA viruses, the SARS-CoV-2 Spike plasmid was transfected to the cells along with a vector control plasmid. Upon completion of 24 h of transfection, the cells were treated with GG1 and GG2 compounds. Samples were scraped after 24 h of treatment, making a total transfection time of 48 h.

4.4.10 RNA isolation, cDNA preparation, and qRT-PCR

The infected or transfected cells were treated with GG1 and GG2 for 24 h, and samples were scraped and washed with 1 X phosphate buffered saline (PBS) for isolation of total RNA using TRIzol reagent [52]. Further, cDNA synthesis was performed using a synthesis kit following the manufacturer's protocol. Synthesized cDNA was used to perform qRT-PCR to assess the expression of specific markers using SYBR green master mix. The relative mRNA expression was assessed using different primers, namely *pcna*, *pRb*, *survivin*, *Bax*, *ccnd1*, *ccnb1*,

cdk1, *apaf1*, *ebna1*, and *spike*. A reference gene, namely, human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was used to monitor changes in gene expression through melt curve analysis.

4.4.11 Cell proliferation assay

To assess the cell proliferation upon GG1 and GG2 treatment, the crystal violet staining method was employed. Upon completion of infection and treatment, the cells were subjected to crystal violet stain according to a protocol as described previously [53].

4.4.12 EB-AO staining

Cell death analysis to study apoptotic, necrotic, and live cells was performed using Ethidium bromide/ Acridine orange (EB/AO) staining at a concentration of 100 µg/mL each for 5 min at 37 °C, followed by PBS washing [54]. Imaging was performed using a fluorescence microscope (Olympus IX83, Olympus, Tokyo, Japan) at 20x magnification. A total of 1000 cells were counted to analyse the percentage of live, apoptotic, and necrotic cells.

4.4.13 Bacterial culture and Antibacterial activity

To assess the antibacterial activity of the hydrogels, a Gram-negative bacterium, *Escherichia coli*, and a Gram-positive bacterium, *Bacillus subtilis*, were used in the current study. Both bacteria were cultured in Luria Bertini agar and incubated at 37 °C for 12 h. The single colonies were picked and cultured in LB broth overnight in a shaker incubator at 220 rpm and 37 °C [55]. The antibacterial activity was assessed by counting viable microbial colonies. The bacterial pellet was collected by centrifuging at 2500 rpm for 5 min and subsequently washed with 1X PBS to achieve a bacterial CFU of 10^6 CFU/mL. Both bacteria were resuspended in 1 mL LB broth and incubated with 0.5 mM of GG1 and GG2 compounds. The untreated control with LB broth alone was used as a negative control. After 1 h incubation, the bacterial suspensions were serially diluted, and a 10^{-6} dilution was spread onto

agar plates to assess antibacterial efficacy [22]. The viable colonies were manually counted, and the percentage of viable cells was plotted using GraphPad Prism.

4.4.14 Statistical analyses

All the experiments were performed in triplicate, and an unpaired t-test was performed to assess statistical significance. The p-value determined the level of significance, wherein p-values < 0.05 , < 0.01 , and < 0.001 were considered statistically significant with 95%, 99%, and 99.9%, respectively.

4.4.15 Characterization Methods

a) UV-visible spectroscopy- UV-visible spectra of the hydrogels, GG1 and GG2, were recorded using a 1 cm path length cuvette. Titration spectra were performed upon successive addition of GMP to an aqueous solution of GSH and GSSG, respectively at a variable mole ratio.

b) Field Emission Scanning Electron Microscopy and Transmission Electron Microscopy (FESEM and TEM)- The hydrogels were diluted up to 500 times in Milli Q water, and 10 μl of it was drop-casted on a clean coverslip and left for air drying for 24 hours. The drop-casted samples were then coated with Au and imaged under the electron microscope.

c) Confocal Microscopy- For the confocal imaging of the samples, we used a confocal microscope from OLYMPUS, model no. IX-83. The Multiline Ar lasers (gas laser) with an excitation wavelength of 405 nm was used to monitor ThT incorporated peptide-nucleotide self-assembled structures. The concentration of ThT was maintained at 5 μM for all the samples. 10 μl sample was drop-casted on a clean coverslip and kept in a vacuum desiccator to remove the solvent. ImageJ software was used to process all the images with linear adjustments of brightness and contrast. All imaging studies were performed at room temperature.

d) Nuclear Magnetic Resonance (NMR)- The NMR studies were performed for GMP, GG1 and GG2 at similar concentrations as for hydrogel (100 mM) using D₂O as the deuterated solvent. To study the NMR of the hydrogel, it was converted to sol owing to its thermo-reversible property and then transferred into the NMR tube.

e) Powder-XRD and FTIR. The hydrogels were lyophilized to obtain the xerogel. Then P-XRD and FTIR were measured for the xerogels. Dry powder of GSH, GSSG, and GMP were used for FTIR studies.

f) Rheological Studies - Rheological studies were performed at room temperature (25 °C) using a parallel plate (25 mm diameter). A small amount of the gel was scooped out with the help of a spatula and kept on the plate for measurement. The strain sweep measurements of the hydrogels were carried out at a constant frequency (10 rad s⁻¹). The frequency sweep studies were performed in a 0.1–500 rad s⁻¹ frequency range maintaining a constant strain of 5%. For the thixotropic loop studies, the strain was varied from 1 to 500% at an applied frequency of 10 rad s⁻¹. Successive low and high strains were applied at a period of 100 seconds for several cycles.

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Chapter 5

Conclusion and Future Prospects

5.1 Conclusion

The spontaneous organization of simple biomolecular building blocks into architecturally sophisticated nanostructures lies at the core of many functional systems in nature. This thesis investigated the intrinsic ability of purine nucleotides to serve as low molecular weight gelators, forming a robust three-dimensional supramolecular hydrogel network. Through metal coordination and interactions with biogenic components, these systems self-assemble into higher-order nanofibrous architectures, mirroring the organizational elegance of biological soft tissues. Among them, GMP emerged as a versatile building block, owing to the formation of higher-order G-assemblies via hydrogen bonding, π - π stacking, and other non-covalent interactions. In this context, the gelation ability of AMP is relatively less explored. This thesis demonstrated the potential of AMP-based hydrogel as a versatile, multifunctional platform.

A key strength of this thesis lies in the development of a facile and straightforward synthetic strategy that is devoid of any chemical modification. This approach ensures the preservation of the native form of biomolecules while minimizing synthetic complexity, cost, and other tedious experimental procedures. Notably, by retention of the pristine form of the biomolecules, their inherent biological functionality is effectively translated into the resulting material. The simplicity of these systems can also provide clearer mechanistic insight into the fundamental process governing the self-assembly. These nucleotide-based hydrogel has the potential to be utilized as a versatile template for several biological and biomedical applications and can replace conventional polymeric gelators.

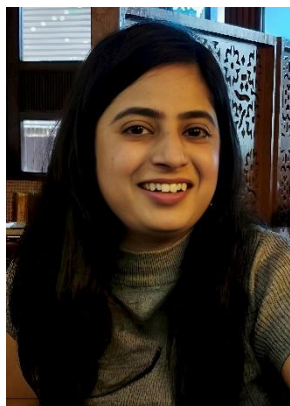
The first three research work compiled in chapter 2 and chapter 3 of the thesis demonstrated the ability of AMP to serve as LMWG to

fabricate a stable, robust and biocompatible hydrogel. The functional group interactions and morphological aspects were investigated by using several spectroscopic and microscopic techniques. The multifunctionality of the Co-AMP hydrogel was explored for a variety of applications. It was used as a biomimetic system for mimicking catalase and peroxidase, and carbonic anhydrase enzymes. The carbonic anhydrase mimicking activity of Co-AMP hydrogel was further used for removal of toxic metals via biomineralization. Interestingly, it was established as an efficient bifunctional electrocatalyst for alkaline water splitting. In the last work, we proposed that a small aliphatic tripeptide glutathione can self-assemble with GMP to form supramolecular hydrogels. We also investigated the morphological evolution for the nucleotide governed formation of entangled fibrillar network. The hydrogels showed intrinsic multimodal bioactivities. In summary, this thesis underscores the importance of these nucleotide-based hydrogels and the proposed facile synthetic strategy adds to their practical implications.

5.2 Future Prospects

Nucleotides, being inherently biocompatible and structurally versatile low molecular weight gelators (LMWGs), offer a compelling platform for the design of adaptive, multifunctional soft materials. Their ability to coordinate with metal ions and several non-covalent interactions endows them with remarkable potential. This thesis has highlighted how AMP and GMP can drive the formation of higher-order multifunctional hydrogels using a simple synthetic strategy. The integration of inorganic and organic motifs to yield hybrid materials presents an opportunity to design biomaterials with desired properties. The dynamic features like stimuli-responsiveness, injectability, and self-healing add to their applicability. These responsive, biocompatible, and biodegradable smart materials hold considerable promise for biomedical applications such as tissue scaffolds, universal delivery platforms, implantable biosensors, and 3D bioprinting technologies. As per the statistics, the exponential upsurge in gel research clearly indicates the

importance of gels as an important class of soft materials in the upcoming era. In this context, the strategy presented herein offers distinct advantages in terms of its simplicity, cost-effectiveness, and wide landscape of available crosslinkers. Moreover, the preservation of native biomolecules in the resulting biomaterials, coupled with their intrinsic multifunctionality, positions these hybrid materials as a versatile interdisciplinary platform. The versatile applications in a single matrix of Co-AMP hydrogel represent endless underexplored opportunities to harness AMP as a gelator for the rational design of functionally diverse soft materials. Apart from the works highlighted in the thesis, we are also investigating the gelation ability of AMP with other metal ions and small-molecule crosslinkers. In the broader landscape of supramolecular soft materials, nucleotide-derived hydrogels stand out as minimalist yet highly functional systems. Despite significant progress, a deeper molecular-level understanding is crucial for enabling the precise design of the next generation of advanced biocompatible soft materials.



D.O.B. 2nd May, 1996

Ms. Vidhi Agarwal was born in Ghaziabad, Uttar Pradesh, India, in 1996. She obtained her Bachelor's in Chemistry (Hons.) from Zakir Husain Delhi College, University of Delhi in 2016, followed by a Master's in Chemistry from CCS University, Meerut in 2018. In January 2020, she commenced her doctoral studies at IIT Indore under the guidance of Dr. Tridib Kumar Sarma. Her research topic was "*Exploring Nucleotides as Low Molecular Weight Gelators to Fabricate Supramolecular Multifunctional Hydrogels for Versatile Applications.*" This thesis represents her modest contribution within the expansive realm of scientific research during the last five years.