Synthesis and Characterization of Supramolecular Hydrogels Using ATP and GMP as Molecular Building Blocks

M.Sc. Thesis

By

MOHD FARHAN ANSARI

DISCIPLINE OF CHEMISTRY

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Synthesis and Characterization of Supramolecular Hydrogels Using ATP and GMP as Molecular Building Blocks

A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree
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Mohd Farhan Ansari

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INDORE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Synthesis and characterization of supramolecular hydrogels using ATP and GMP as molecular building blocks"** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF CHEMISTRY, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2020 to June 2021 under the supervision of **Dr. TRIDIB KUMAR SARMA**, Assistant 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.

M. Farhan
09/06/2021

Mohd Farhan Ansari

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

Tridib K. Sarma

(Dr. TRIDIB KUMAR SARMA)

Mohd Farhan Ansari has successfully given his M.Sc. Oral Examination held on **9th June 2021.**

Tridib K. Sarma 09-06-21

Signature of Supervisor of MSc thesis

Convener, DPGC

10.06.2021

Signature of PSPC Member 1

9.6.2021

Signature of PSPC Member 2

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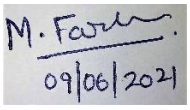

Mohd Farhan Ansari

M.Sc. 2nd year

Dedicated to

My Family

Abstract

Molecular self-assembly is the fundamental basis of supramolecular chemistry. Supramolecular chemistry, also known as “chemistry beyond the molecule”, deals with the study of the formation of ordered, directional, tunable, reversible molecular aggregates or organizations by noncovalent interactions (pi stacking interactions, van der Waals, H-bonding metal coordination, solvophobic, electrostatic, etc.). One of the important applications of supramolecular self-assembly is the synthesis of semi-solid colloidal “hydrogel”. Hydrogels are soft in nature which resembles biological tissues. They have ability to absorb a large amount of solvent in their matrix, making them attractive biomaterials for various applications in developing targeted drug delivery system, tissue engineering, wound healing, catalysis, etc. The three-dimensional architecture of hydrogel is based on structural properties of gelator molecules which form cross-linked networks through above mentioned weak non-covalent interactions that can hold a large volume of water. The native biomolecules such as peptides, nucleic acids, and carbohydrates are also used as an exclusive class of small gelator molecules. Presence of various noncovalent interaction sites makes the nucleotides a naturally occurring hydrogelators. The stimuli responsiveness and biocompatibility of the resultant supramolecular self-assembled hydrogels can be harnessed towards fascinating functional materials taking advantage of the inherent characteristics. Herein, we report the synthesis of a novel supramolecular self-assembled hydrogel by coordinating nucleotide ATP with GMP. The ATP-GMP hydrogel was formed by simple mixing in the molar ratio of 1:1. The hydrogel is expected to exhibit various fascinating properties such as synthetic reversibility, self-healing, self-supporting, injectable, bio-mimicking, stimuli responsiveness, ion-conducting, and significant water holding ability that might influence a wide range of applications in material as we as biomedical sciences. This thesis covers the following topics: introduction and background, experimental section, and results and discussions.

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two pebbles with total 81 g weight, (d) three pebbles with total 122 g weight, and (e) finally when all the weight was removed.

Figure 9: Digital images ATP-GMP hydrogel showing (a) thermal responsiveness and (b) pH responsiveness.

ACRONYMS

ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
GMP	Guanosine Monophosphate
GTP	Guanosine Triphosphate
CTP	Cytosine Triphosphate
cGMP	cyclic Guanosine Monophosphate
cAMP	cyclic Adenosine Monophosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
FMN	Flavin mononucleotide
FAD	Flavin Adenine Dinucleotide
NAD	Nicotinamide adenine dinucleotide
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscopy
AFM	Atomic Force Microscopy
DSC	Dynamic Scanning Calorimetry
NMR	Nuclear Magnetic Resonance
XRD	X-ray Diffraction
CD	Circular Dichroism
UV-Vis	Ultraviolet-visible
3D	Three-dimensional
pH	Potential of hydrogen

Chapter 1
Introduction and background

1.1 Introduction

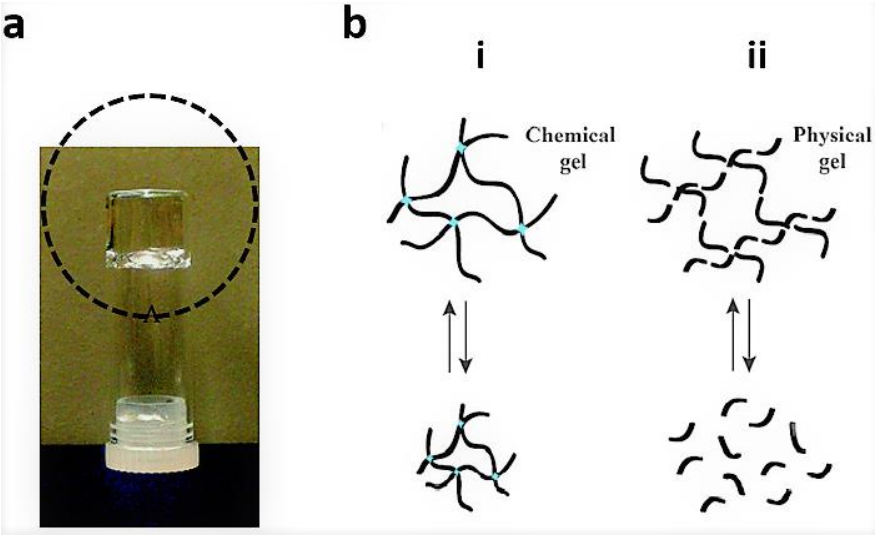
Self-assembly at the molecular level of organization is a widespread phenomenon in both chemicals as well as biochemical world which describes the spontaneous organization (without external control) of molecules into ordered aggregates and is believed to play a crucial role in the growth, sustenance, and advancement of life.[1-3] Molecular self-assembly is the fundamental basis of supramolecular chemistry. Supramolecular chemistry, also known as "chemistry beyond the molecule", deals with the study of the formation of ordered, directional, tunable, reversible molecular aggregates or organizations by noncovalent interactions (pi stacking interactions, van der Waals, H-bonding metal coordination, solvophobic, electrostatic, etc.). Such interactions are responsible for self-assembly due to their very low energies and often require no activation energy. [4] Over the past couple of decades, material chemists have been motivated to use the principles of nature's self-assembly and supramolecular chemistry to formulate artificial biomaterials, with a high level of precision and dynamics, to achieve functions such as recognition, adaptation, stimuli responsiveness, transport, and catalysis. [5] With the immense development in nanotechnology, Supramolecular chemistry has found several applications in formulating nano biomaterials including drug delivery carriers, hydrogels for tissue repair cell and culture, micelles, crown ethers, etc. However, in recent years supramolecular gels have attracted immense interest from chemists due to their potential applications suggested till now in fields including diagnostics, tissue scaffolding, catalysis, and targeted drug release biomedicine, etc.[6] Supramolecular gels are characterized by nanostructured soft colloidal materials with viscoelastic properties. These gels are formed by a continuous three-dimensional cross-linked network of low-molecular-weight organic compounds by the action of intermolecular non-covalent interactions. The continuous three-dimensional cross-linked network structure of gel is due to the coexistence of two distinct phases: a large amount of "liquid" phase and a comparatively small amount of a "solid" phase which

form the crosslinked network and restricts the flow of the liquid phase. [7-8] Based on the solvents entrapped as liquid phase or solvents in which they form gels, supramolecular gels are further classified as organogels (organic "solvent" is used as the liquid phase), and hydrogels ("water" is as used as the liquid phase).[9-10] Since water is the unique solvent that maintains all forms of life on earth, thus due to the presence of a large amount of water, supramolecular hydrogels are considered a relatively simple heterogeneous system that surprisingly has a broad range of applications in life science. Supramolecular hydrogels are formed due to the hierarchically built-up of small molecules from the molecular into the supramolecular level. Thus, all the information needed for supramolecular self-assembly and function is programmed at the molecular level by selecting an appropriate organic solid phase moiety.

1.2 Gels: Introduction

A gel is a semi-solid or nonfluid colloidal state of matter comprising of a continuous three-dimensional cross-linked network, characterized by viscoelastic properties with entrapped solvent inside their 3D cross-linked network.[11] The cross-linked skeleton of the gel is due to the simultaneous existence of two different phases: a large amount of "liquid-like" dispersed phase and a comparatively smaller amount of "solid-like" network of dispersion medium which restricts the bulk flow of the liquid phase. In this way, gels are the dispersion of liquid molecules within a solid phase.[12] The unique and substantial quantities of these two components give them dynamic viscoelastic properties ranging from soft to hard and from weak to tough. Gels exhibit no flow when in a steady state. A gel is visually inspected by a very simple and classical "inverting-vial" method, where the gel is observed by "naked eyes" just by flipping the vial upside down.[13] The gel is showing stability under the gravitational pull (Figure 1 a). The self-assembly process of the small molecules resulting in the formation of a gel is known as gelation, and a subset of these small molecules forming gel is called gelators. Further, based on this gelation process, these gels can be grouped into two different classes; physical gels which are reversible in nature as they are obtained by the means of weak non-covalent interactions. The second class is of chemical gels which are irreversible

in nature as they are formed employing strong covalent interactions between the gelators (Figure 1 b).



. ***Figure 1.*** *(a) Representation of a Gel. [Adapted from reference 64] (b) Fibrous network of (i) chemical gel showing covalent crosslinking and (ii) physical gel showing reversible non-covalent associations. [Adapted from reference 65]*

1.3 Types of Gels

On the basis of their constitution, gels can be classified into two different categories: polymeric gels (also known as macromolecular gels) and supramolecular gels. The polymeric gels are characterized by a soft and/or solid-like 3-D network structure formed by the aggregation of one or more than one polymer (or macromolecular compound) by virtue of interactions in liquid medium. The 3D network structure of polymeric gels enables them to preserve a large amount of solvent(s). The polymeric gels exhibit a large number of characteristic properties among which some of the most important properties are: High mechanical strength and irreversible in nature.[14] The second category of gel is supramolecular gels which are derived from the aggregation of comparatively small gelator molecules(low molecular mass compounds) by virtue of the combination of non-covalent interactions like van der Waals interactions, donor-acceptor interactions, pi-pi stacking, and H-bonding, etc.[15]These are reversible gels.

1.4 Supramolecular hydrogels derived from nucleobases and nucleotides

There are a large number of gelators such as organic, inorganic, polymer, peptides, nucleic acid, polysaccharides, etc based gelators have been studied till today**.** In this project work, we mainly focus on the synthesis of supramolecular hydrogels using nucleotide (ATP and GMP) as the molecular building blocks and discussing their potential applications**.** Natural nucleobases are nitrogen-containing heterocycles such as purine and pyrimidine that are basic biological building units of nucleosides, nucleotides, and nucleic acids. RNA and DNA are the two main classes of nucleic acids which act as the master blueprint of life and carry the genetic information of all free-living organism and most viruses. *[16-17]* The nucleotides also play several key roles in metabolism at the cellular level such as providing chemical energy (in the form of ATP, ADP, GTP, CTP, etc), participating in cell signalling (by cGMP, cAMP), and acting as an important cofactor in enzymatic reactions (FMN, FAD, NAD, $NADP^+$, etc). These nucleobases contain different organic functional moieties such as hydroxy, carboxylic acid, carbonyl, phosphates, aromatic rings which are potentially involved in the formation of different noncovalent interactions which act as the key factor for supramolecular self-assembly (Figure 2).*[18]* Among all the nitrogenous bases, guanine and its derivatives have received the greatest interest of chemists as a fascinating chemical tool to create a supramolecular network. This is due to their unusual property of showing different fashion of self-assembly in different conditions to form entirely different supramolecular macrostructures such as ribbon/band-like structure (simply referred to as G-ribbons), planar quartet structure in the presence of mono or divalent metal ions (simply referred to as G-quartets) where the four guanine bases are arranged through cyclic hydrogen bonds to form a planar macrocyclic structure, and many more. This unusual self-assembly pattern of guanosine and its derivatives is mainly reflected in two hydrogen bond donors (N1 amide and N2 amino) and two acceptors (N7 and O6).*[19]* Further, the planar G-quartets undergo π-π stacking to form octamers or a higher degree of three-dimensional helically stalked aggregates known as quadruplexes. Due to such unique properties, the supramolecular self-assembly of guanine and its

derivatives has received tremendous attention for their future applications in medicines, material sciences, nanotechnology, and supramolecular chemistry fields.[20]

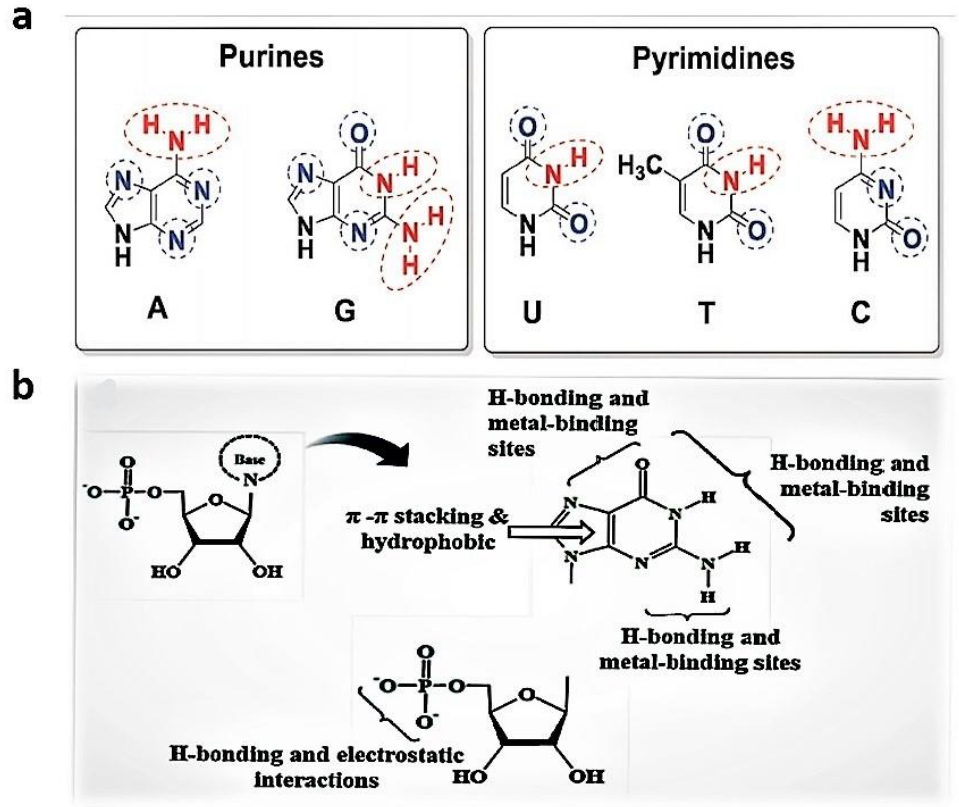


Figure 2. *Illustration of basic structures of [Adapted from reference 17] (a) nucleobases showing hydrogen bond donors (red) and acceptors (blue) sites, and (b) nucleotides showing different non-covalent interaction sites.*

1.5 Hydrogels derived from Adenosine nucleotide and GMP

In this project work, we mainly focus on the synthesis of supramolecular hydrogels using GMP with ATP/ADP/AMP as the molecular building blocks and discussing the chemical and physical properties of ATP useful for the synthesis of ATP-GMP hybrid hydrogel and to study their stimuli-responsiveness. The nucleotide skeleton consists of different chemical moieties such as heteroatom residues of nitrogenous bases, phosphate residue, and aromatic rings. These moieties are involved in different types of non-covalent interactions depending on the counterpart present in the self-assembly process.

These moieties mainly affect the steric and electrostatic environment which alters the noncovalent interactions. For example, moving from AMP to ADP to ATP, the number and size of the anionic phosphate group increases which affects the stacking interaction between these nucleotides. It is noteworthy that in the case of supramolecular metallogels (such as Ag^+ metallogels), gelation occurs only with the AMP (or GMP) and cannot form from ADT and ATP even by adjusting the pH and concentration of the system. This observation shows that the metal ions are not coordinated with the phosphate-binding groups.[21] However, in this project where we have demonstrated the synthesis of the hydrogel by interacting GMP with AMP/ADP/ATP, a very contrasting result was observed as obtained in the case of metallogel synthesis. It is noteworthy that AMP did not form hydrogel even by adjusting the temperature and concentration, ADT formed hydrogel only at low temperature whereas ATP formed a stable hydrogel even at room temperature. This observation clearly shows that GMP mainly coordinated with the phosphate-binding groups. In addition, several other interactions including van der Waals force, solvophobic interaction, hydrogen bonding may also participate in the mechanism to provide reversibility, tunability to this self-assembly process. In recent years chemists have mainly focused on formulating biocompatible supramolecular hydrogel using ATP building blocks. Adenosine triphosphate (ATP) is a known biological energy currency that drives various non-spontaneous processes in the cellular environment. It is one of the naturally available supramolecular synthons which show diverse non-covalent interactions such as hydrophobic interactions (adenine), electrostatic interaction (phosphate group), and H-bonding (adenine and ribose). In addition, the synthesis and degradation of ATP can be performed by many naturally occurring enzymes, and hence the ATP induced or ATP driven supramolecular systems can be used to impart the enzymatic strategies and stimuli responsiveness to design artificial systems with biomimetic activities.[22]

1.6 Applications of Supramolecular Gels

Supramolecular hydrogels are well defined by several unique and characteristic properties such as capability of trapping large quantity of water or solvent, self-healing, dynamic stimuli responsiveness, mild processing conditions, etc. These properties make the hydrogels well suited for several biological and biomedical applications. In recent few years, on the basis of their unique architecture with the unconventional properties lying between the domains of conventional solids and liquids, and the increased understanding of protein functioning and structural biology, chemists have put significant efforts on the incorporation of biomolecules as the functional motifs on supramolecular hydrogelators for their biological and biomedical applications. [23-25] Some of the notable biomedical applications of the hydrogels are tissue engineering,[26] controlled and targeted drug delivery,[27-29] wound healing,[30-31] immunomodulation, etc (Figure 1.3). As the supramolecular hydrogels are formed by means of weak non-covalent interactions, they have witnessed many applications as biomaterial,[14-32] adhesive,[33] in water treatment and pollution mediators,[34] and actuators.[35] In Addition, they also possess remarkable strength as well as elasticity, they become valuable for load-bearing biomedical applications such as in cartilage and skin.[36] There are several classes of supramolecular hydrogels based on the constituent molecules which also broaden their application in many interdisciplinary fields of science. One of such class is supramolecular metallogel, which are formed by incorporation of metal ion(s) with the gelator molecules by means by various intermolecular covalent or non-covalent interactions such as H-bonding, hydrophobic, and π-π stacking.[37] These Supramolecular metallogels are considered to be promptly responsive and stimuli-sensitive towards chemical stimuli which infer their sensing applications.[38-42]Also, the various interactions of metal ions with gelator molecules lead to several fascinating properties, such as optical, electronic, colour, magnetic, and redox responsive behaviour.[43-44] Such fascinating properties of metallogels make them a multifunctional material for various developing fields of sciences for the applications such as enzyme-mimetics, ion-conductance, adsorption of gas, and catalysis.[45-47]

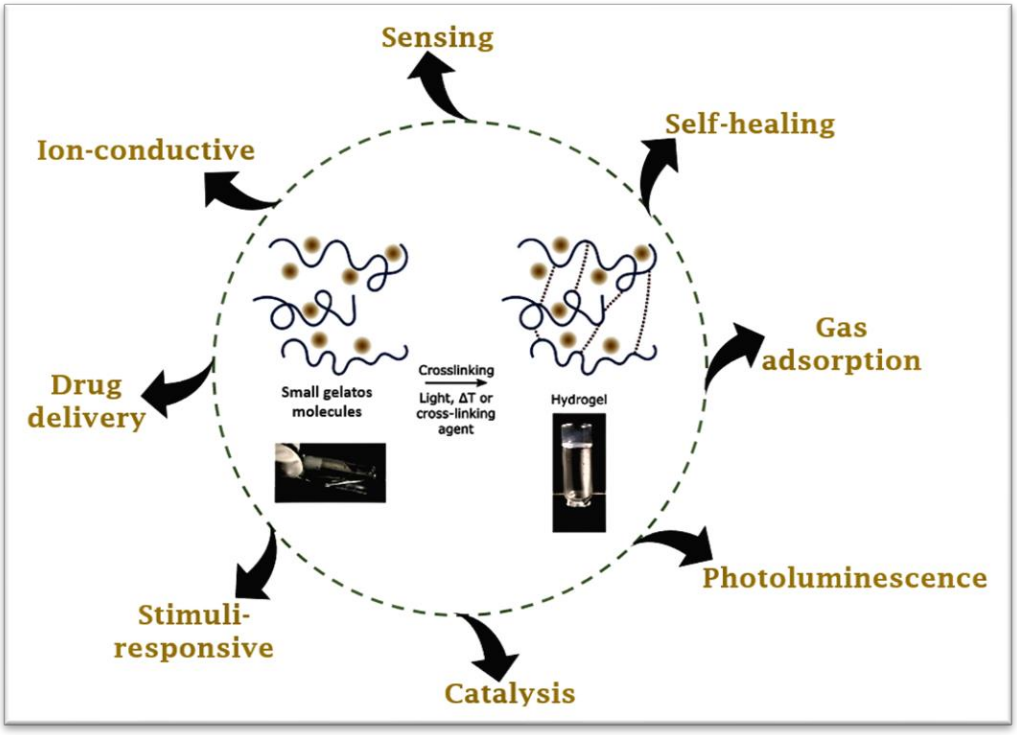


Figure 3. *Schematic representation of hydrogel synthesis and their diverse applications.*

1.7 Characterization techniques

Recent advancement in the understanding of supramolecular self-assembly and substantial increase in number of hydrogelators at both nanoscale and molecular levels require more realistic and faithful characterization of hydrogels. Among various known characterization techniques only those methods are preferred that preserves the fundamental structural properties of hydrogels. The primary objective of characterization and analysis of hydrogels is to improve understanding how the small gelator molecules are interacted and arranged in to a 3D structure, leading to develop new and practical ideas for the development of various supramolecular materials useful in industrial as well as biomedical applications. Some of the important characterization techniques are visual inspection, microscopy (including SEM and TEM), rheology, differential scanning calorimetry (DSC), X-ray diffraction (XRD), circular dichroism (CD), UV-vis characterization, etc.

1.7.1 Visual Inspection

A gel is visually inspected by a very simple and classical "inverting-vial" method, where the gel is observed by "naked eyes" just by flipping the vial upside down. The gel is showing stability under the gravitational pull.

1.7.2 Microscopy

The supramolecular hydrogels usually exhibit 3D network structure of micrometer and/or nanometer dimensions. With the rapid development in various microscopic techniques, they are used as primary characterization techniques to study the morphology of hydrogels. Among various known microscopic techniques, the frequently techniques include scanning force microscopy (SFM) or atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), etc. Several cryogenic techniques are also used to study the structure of hydrogel at cryogenic temperature (liquid nitrogen temperature). Microscopic techniques are used to achieve significantly very high resolution. For example, transmission electron microscopy can achieve resolution of more than 50 pm.[48] In addition, a new microscopic techniques have been developed, environmental scanning electron microscopy (ESEM) used to characterized hydrogels under a particular humidity condition.[49-50]

1.7.3 Rheological characterization

Rheological technique is used to assess the mechanical strength of the gels. Elastic nature of the gel is evaluated by comparing the magnitude of loss modulus (G´´) and storage modulus (G´) in strain and frequency sweep measurements. Also self-recovery characteristic of the gel characterized by performing thixotropic loop test.[51]

1.7.4 Differential scanning calorimetry (DSC)

It is an important thermo-analytical technique used for the characterization of the hydrogels. This method is based on the principle of measuring the difference in the amount of heat needed to raise the temperature of a sample and reference

as a function of temperature. Throughout the experiment, nearly same temperature is maintained for both the sample and the reference. This method is used to determine the gelation temperature (T_{gel}), which gives the information of the point where the non-covalent interactions are broken by heat energy. [52]

1.7.5 X-ray powder diffraction (XRD)

X-ray powder diffraction (XRD) is a frequently used characterization technique for supramolecular hydrogels especially in case of microcrystal formation during gelation. XRD mainly explains the molecular organization and nano architect of hydrogels. [53]

1.7.6 Circular dichroism (CD)

Circular dichroism (CD) is one the important characterization techniques with various important applications including study the secondary structures of proteins. In supramolecular hydrogels, CD is frequently used to study self-assembled structures at the gel-to-sol transition phase.[54-55] However, using CD alone is not a such precisely informative therefore, it is often combined with other techniques such as UV-Vis to make any precise conclusion on the structural determination of supramolecular hydrogels.

1.7.7 Spectroscopic characterization

Various spectroscopic techniques such as NMR, infrared (IR), UV-Vis, or fluorescence are used as quantitative as well as qualitative measure to study the arrangement of hydrogelators in supramolecular hydrogels. They basically work on the principle of detecting gelator-gelator or gelator-solvent interactions in primary or secondary structures of self-assembled superstructures. UV-vis spectroscopy technique is used to study pi-pi stacking (or aromatic− aromatic interactions) or coordination of metal ions in hydrogelation process.[56-58]It is often combined with CD to study more precise information of arrangement of molecules in hydrogels. Fluorescence is also used as an important characterization technique for the study of the self-assembly between aromatic moieties. Furthermore, NMR spectroscopy can also be used to study the changes in chemical shift values during self-assembly process.[59] It gives the information of transformation of different functional groups present in small gelator molecules in supramolecular sol-to-gel transition process.

Chapter 2
Experimental Section

2.1 Materials

For the studies, all the four nucleotides, Adenosine 5′-triphosphate (ATP) disodium salt hydrate, Adenosine 5′-diphosphate (ADP) disodium salt hydrate, Adenosine 5′-monophosphate (AMP) disodium salt hydrate guanosine-5′-monophosphate (GMP) disodium salt hydrate, Adenosine, and Guanosine were purchased from Sigma-Aldrich. Histidine, Arginine, Acetonitrile, Dimethyl sulfoxide, Ethanol, alkaline phosphate buffer and Hydrochloric acid were purchased from Merck, India. All the chemicals were of analytical grade and were used without any further purification. Milli Q water was used throughout the experiments.

2.2 Instrumentation

UV-visible characterizations were carried out by using a spectrophotometer from Varian Cary 100 Bio. Field emission scanning electron microscopy (FESEM) images were recorded on a Carl Zeiss Supra 55 instrument after coating with Au. The gel samples were prepared in water by using the diluted gel, dropped cast on a glass slide, and dried in vacuum.

2.3 Synthesis of Adenosine nucleotides: GMP Hydrogels

100 mM aqueous solutions of GMP were freshly prepared by dissolving them in 0.5 mL milli-Q in three separate vials (labelling 1, 2, and 3 for ATP, ADP, and AMP respectively). For the synthesis of combination of these three, adenosine nucleotide-GMP hydrogels, 20.36 mg (100 mM) GMP was added to each of the above three vials final volume of 0.5 mL and the molar ratio of 1:1 between adenosine nucleotides and GMP. The resultant mixtures were formed with suspended nucleotide particles. After heating these three mixtures on a room temperature of about 65 ^{0}C for a few minutes, these mixtures get turned into clear colourless solutions. The resultant solutions were kept standing at low temperature ~5 ^{o}C for 30 min to 1 h, upon which the hydrogel formation was observed in vials 1 and 2 only. After that these systems were kept standing at

room temperature for few minutes, the hydrogel formed in vial 2 (ADP-GMP system) starts breaking into solution whereas the hydrogel formed in vial 1 (ATP-GMP system) remains stable. The formation of gel was evidenced by a physical test known as "inversion test" where the flow of gel or liquid in an inverted vial was observed under the influence gravity (Figure 4 a, b, and c).

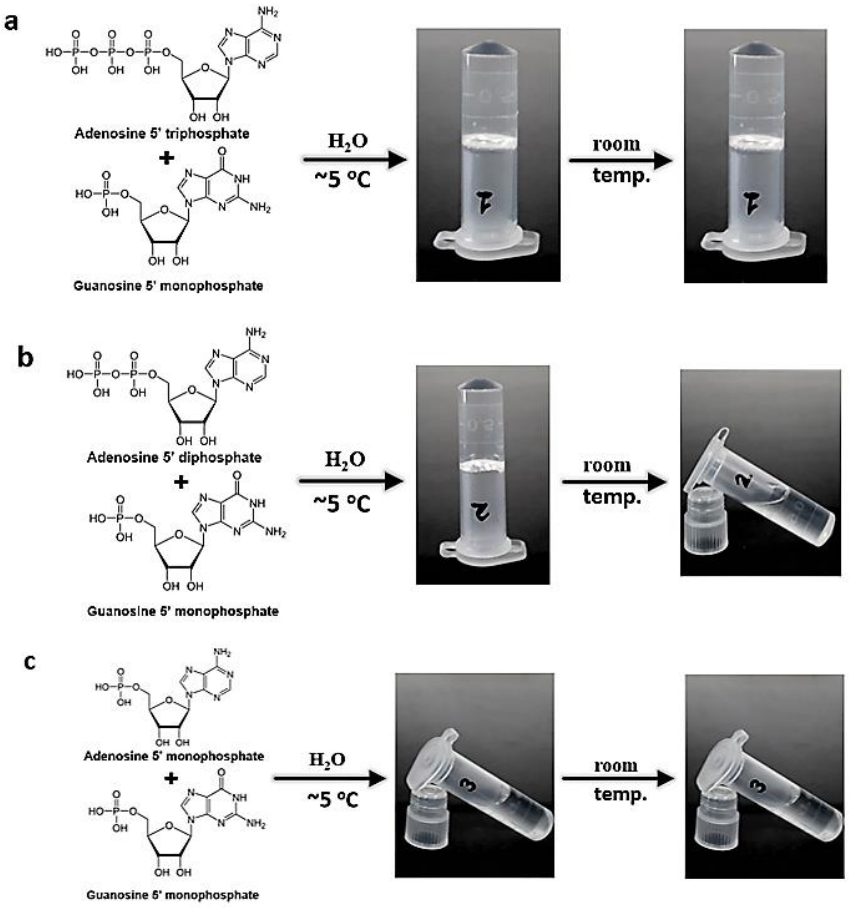


Figure 4. *Steps to synthesize GMP hydrogels with different adenosine nucleotides (a) ATP-GMP gel was formed at low temperature and remains stable at room temperature, (b) ADP-GMP gel was formed at low temperature and stable only at low temperature, and (c) AMP did form gel with GMP even at low temperature.*

Chapter 3
Results and Discussion

3.1 Selection and optimization of nucleotides for hydrogelation

At the beginning of our experiment all the three adenosine nucleotides (ATP, ADP, and AMP) were taken to synthesize the hydrogel with GMP counterpart, maintaining their molar ratio of 1:1. It was observed that among the three combinations (ATP-GMP, ADP-GMP, and AMP-GMP), only ATP formed a stable hydrogel with GMP and it remains stable even at room temperature whereas ADP formed the gel only at low temperature (~ 5 ^{o}C) which was not stable and converted to sol at room temperature. Surprisingly, AMP did not form the gel even at a very low temperature (Figure 4). It was thus observed that the formation of gel with GMP found effected by varying the number of phosphate groups in the adenosine nucleotide. This observation indicates that the phosphate group(s) in the nucleotides play a vital role the gelation process. This moiety mainly affects the steric and electrostatic environment which alters the noncovalent interactions. For example, moving from AMP to ADP to ATP, the number and size of the anionic phosphate group increases which affects the stacking interaction between these nucleotides.[60] Further, the optimum concentration of ATP and GMP require for gelation was studied. The minimum gelation concentration for the molar ratio 1:1 was found to be 100mM by invert vial method (Table 1. a). However, on changing the molar ratio of ATP and GMP, it was found that gelation occurs only when the ATP and GMP have the molar concentration of 75 mM and 100mM, and vice-versa (Table 1. b)

a

	[ATP] (mM)	[GMP] (mM)	[Individual components] (mM)	Result
1A	100	100	50	**Gel formation**
1B	75	75	35.5	Viscous solution
1C	50	50	25	Clear solution
1D	25	25	12.5	Clear solution

b

[ATP] (mM)	[GMP] (mM)	Result
100	25	Clear solution
100	50	Viscous solution
100	75	Viscous solution
75	100	**Gel formation**
50	100	Viscous solution
25	100	Clear solution

Table 1. *Table showing the minimum concentrations of the ATP and GMP for gelation. (a). with 1:1 molar ratio of reactants, (b). with different molar ratio of reactants*.

3.2 Morphology and structural characterization

Recent advancement in the understanding of supramolecular self-assembly and substantial increase in number of hydrogelators at both nanoscale and molecular levels require more realistic and faithful characterization of hydrogels. Among various known characterization techniques only those methods are preferred that preserves the fundamental structural properties of hydrogels.[61] The visual inspection of the hydrogel was done by the simplest classical "inverting-vial" method where the hydrogel is assessed by "naked eyes" just by flipping the vial upside down. The formation of hydrogel was evidenced by showing stability under the gravitational pull. The structural characterization of the ATP-GMP hydrogel was done by using Scanning electron microscopy (SEM) technique, utilizes a beam of accelerated electrons as a source of illumination. The Scanning electron microscopy (SEM) images of the vacuum-dried ATP-GMP hydrogel confirms the formation of entangled three-dimensional web like network structure (Figure 5. a and b), whereas the ADP-GMP hydrogel exhibits highly entangled three-dimensional thread-like network (Figure 5. c and d) and the AMP-GMP hydrogel revealed fused star-like dense fibrillar morphology (Figure 5, e and f).

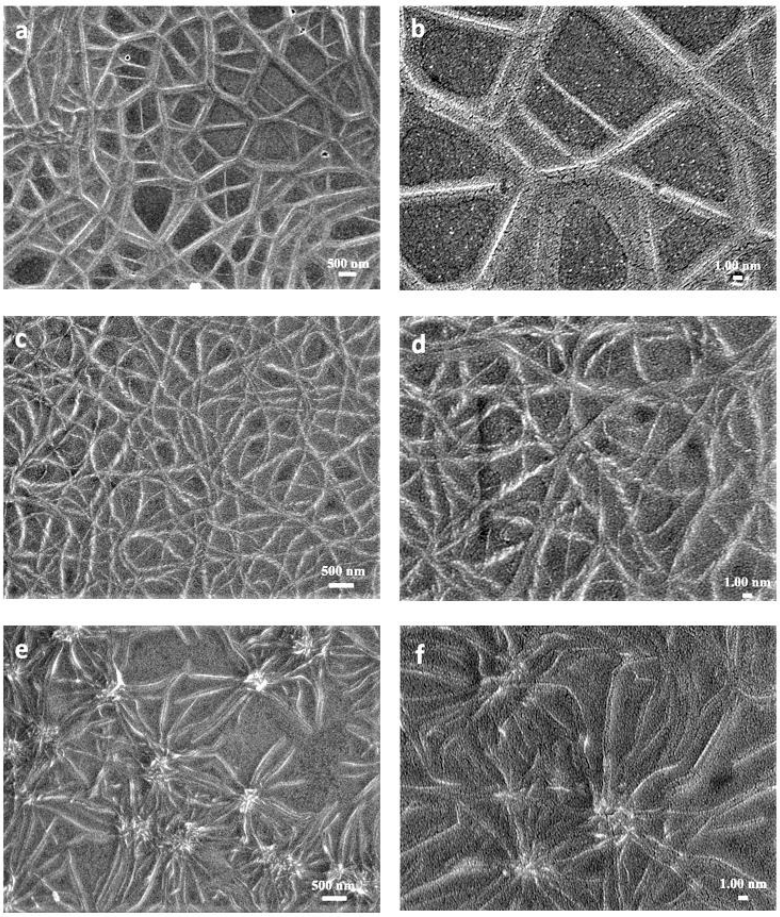


Figure 5. *FESEM images of (a and b) ATP-GMP hydrogel showing dense web-like network, (c and d) ADP-GMP hydrogel showing thread-like entangled network, and (e and f) AMP-GMP hydrogel showing fused star-like fibrillar network.*

3.3 Spectroscopic characterization

UV-Vis characterization

Absorbance of nucleotides in the range of 240-290 nm when irradiated with UV light, is attributed to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. GMP shows maxima at wavelength of 252 nm along with a shoulder at 275nm. ATP shows peak at 259 nm. In case of ATP-GMP gel, absorbance is observed at 257 nm (Figure 6). There is a slight bathochromic shift with respect to GMP and the shoulder band has diminished suggesting interaction between GMP and ATP. For further

insight in the structure, we would require to perform CD and XRD, which couldn't be done due to time constraints.

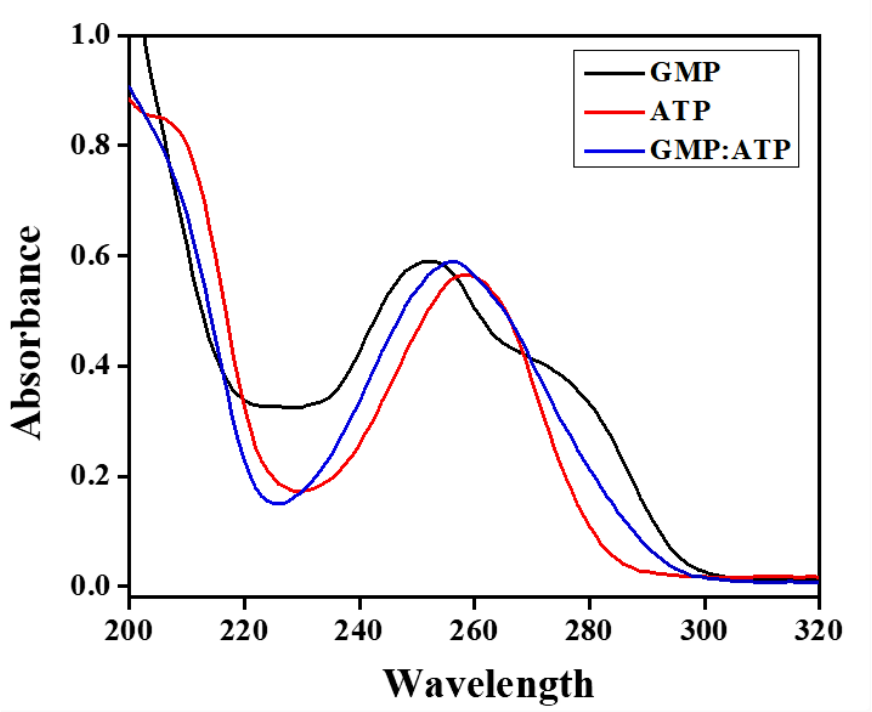


Figure 6. *Bathochromic shift observed in the UV spectrum of GMP upon addition of ATP*

3.4 Self-healing and self-sustaining behavior of hydrogel

Two pieces of ATP-GMP hydrogel were taken on a petri dish. One of these two pieces was incorporated with a dilute solution of phenol red dye (yellow piece) and the other one was incorporated with a dilute solution of malachite green (Blue piece). Then, the two pieces were put together and joined by a mild press and left untouched for a few minutes. It was observed that the bicolored monolithic gel formed could be lifted and suspended in air by holding one side (Figure 7. a). The gel was found stable against the gravitational pull and maintained its wholeness, showing the successful self-healing. The ATP-GMP hydrogel also show remarkable injectability, confirmed by using dye incorporated gels in syringe to write the word 'gel' (Figure 7.b). The self-healing studies with remarkable injectability suggest that the ATP-GMP hydrogel could successfully be used as an injectable soft and flexible hydrogel

system, demonstrated by makings 3D patterns (shaped as word “gel”) using glass syringe needle (Figure 7. b).

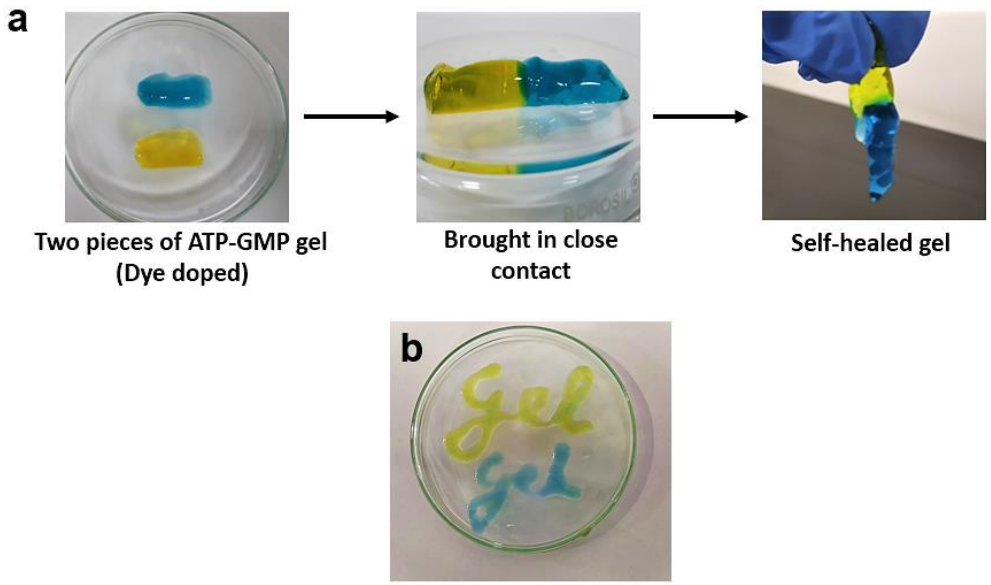


Figure 7. *(a) Digital images showing the self-healing in ATP-GMP hydrogel using phenol red and malachite green dyes, (b) digital image showing the injectability of ATP-GMP hydrogel (dyed with phenol red and malachite green)*

The ATP-GMP hydrogel also shows significant rigidity, tested by taking a small piece of cylinder-shaped hydrogel and three stone pebbles of different masses (36 g, 45 g, and 41g). One by one all the three pebbles were placed over the gel and observed that the gel held its shape up to the total weight of 122 g (Figure 8. a, b, c, d). However, when the applied weight was removed, slight reduction in the height of gel was observed (Figure 8. e). The reduction in height of the gel occurred due to leakage of water as more weight is applied over the gel.

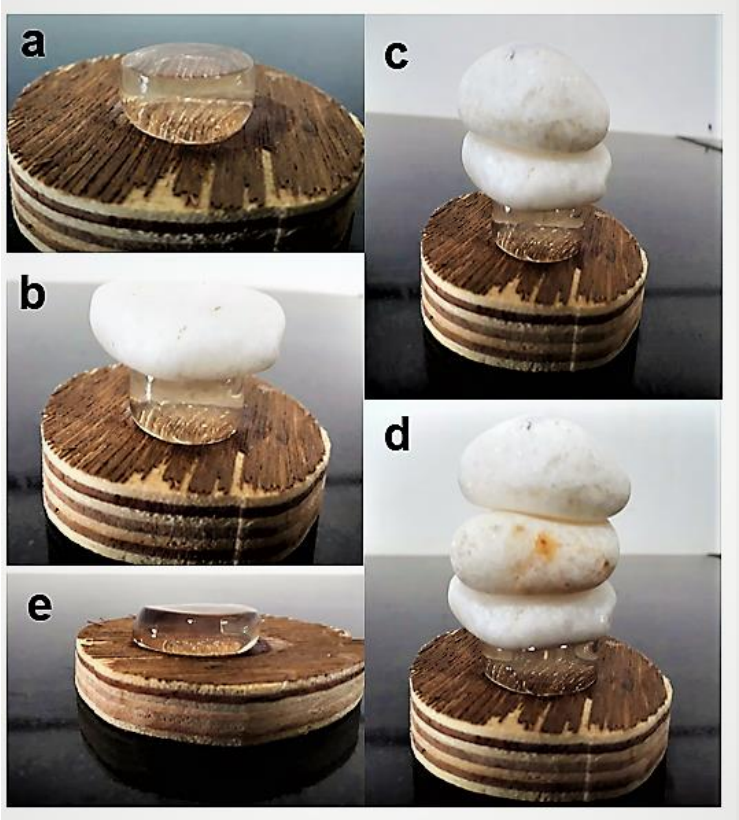


Figure 8. *Digital images showing Self-sustaining behaviour of ATP-GMP hydrogel, (a) initially without any weight (b) one pebble with 36 g weight (c) two pebbles with total 81 g weight, (d) three pebbles with total 122 g weight, and (e) finally when all the weight was removed.*

3.5 Stimuli responsiveness of hydrogel

Hydrogels are formed as a result of self-assembly of small hydrogelator molecules through weak non-covalent interactions which can often be altered upon exposure to various physical and chemical stimuli. The remarkable structural properties of the ATP and GMP nucleotides with different functional moieties such as aromatic ring, phosphate groups, and nitrogen and oxygen containing nitrogenous bases make them responsive toward various stimuli such as pH, temperature, etc. The responsiveness of hydrogel to various stimuli and their reversibility finds several important applications in developing a dynamic drug delivery system.[62-63] At first, thermo responsive nature of ATP-GMP hydrogel was studied. It was observed that the hydrogel is stable at room temperature and collapsed to sol when the temperature was increased. Further,

the pH sensitivity of the hydrogel was studied by using basic amino acids namely arginine and histidine, and was found to be unstable in the presence of these two amino acids. On addition of these two amino acids, the hydrogel was completely converted to sol (Figure 9).

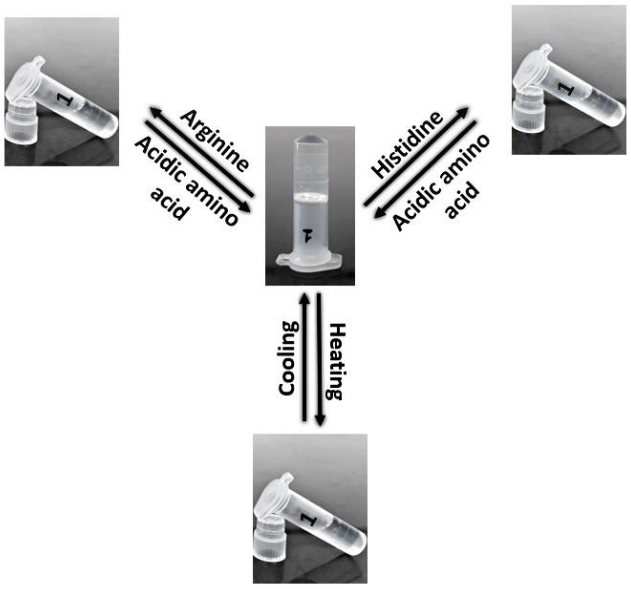


Figure 9. *Digital images ATP-GMP hydrogel showing (a) thermal responsiveness and (b) pH responsiveness*

CONCLUSION

In summary, adenosine 5' triphosphate (ATP) is commercially available trinucleotide well suited for dynamic supramolecular self-assembly with guanosine 5' monophosphate (GMP) to produce a multifunctional hydrogel. The hydrogel was synthesized simply by mixing two gelator components in aqueous medium followed by simple heating. The simple synthetic approach without involving any external stimuli such as significant heating/cooling, use of specific chemical reagent or ultrasonication, reveals the effective ability of these small natural biomolecules to change into a supramolecular self-assembled structure through weak non-covalent interactions. These weak interactions include hydrogen bonding, π-π interactions between aromatic rings as well as van der waals interactions constructively combine to form the self-assembled fibrillar nano-hybrid ATP-GMP hydrogel, attributed by the spectroscopic studies. During experimental works, it was observed that GMP form a stable hydrogel selectively with ATP only. However, ADP formed the hydrogen but was found unstable at room temperature whereas AMP did not form hydrogen even at low temperature. This observation indicates that the phosphate group(s) in the nucleotides play a vital role the gelation process. This moiety mainly affects the steric and electrostatic environment which alters the noncovalent interactions. It was also observed that ATP-GMP formed stable hydrogel at the molar ratio of 1:1 with concentration of 100 mM. The SEM images of the dried ATP-GMP hydrogel confirms the formation of entangled three-dimensional web like fibrous network to hold the bulk solvent molecules within the gel matrix. The hydrogel also showed remarkable self-healing and injectability. In spite of being soft and flexible, the hydrogel showed good mechanical stability. The ATP-GMP hydrogel also evidenced stimuli responsiveness toward temperature, pH, etc. It was observed that the hydrogel is stable at room temperature and collapsed to sol when the temperature was increased. Further, the pH sensitivity of the hydrogel was studied by using basic amino acids namely arginine and histidine, and was found to be unstable in the presence of these two amino acids. On addition of these two amino acids, the hydrogel was completely converted to sol. With the astonishing multifunctional

properties and simple synthetic approach, the ATP-GMP hydrogels might find various future applications in the development of smart biomaterials, targeted drug delivery systems, and separation techniques.

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