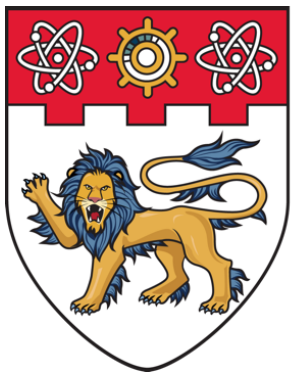

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

SYNTHESIS AND CHARACTERIZATION OF UREIDOPYRIMIDINONE SUBSTITUTED GELATIN BASED ELASTOMERIC HYDROGEL

AMIT PANWAR

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2019

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AMIT PANWAR

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

22nd August 2019

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Date

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Amit Panwar

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Associate Professor Tan Lay Poh

Authorship Attribution Statement

This thesis material is not published yet in any peer reviewed journal.

The contributions to the project work are as follows:

- Associate professor Tan Lay Poh has supervised the project work and have guided the direction of the project.
- I did all the experiments and characterization for my project work.
- I performed all the laboratory work at the School of Materials Science and Engineering, Singapore and analyzed the data myself.
- All microscopy, including sample preparation, was conducted by me in the Facility for Analysis, Characterization, Testing and Simulation.

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Abstract

Elastomeric hydrogels have potential applications in implantable stretchable electronics devices including heart sensors/monitors, drug patches etc. Synthetic polymers polyacrylamide and hybrid gels have been used for the fabrication of stretchable electronics device fabrication. Replacement of synthetic polymer elastomeric hydrogels with natural protein based elastomeric hydrogels would provide better biocompatibility, biodegradability and sustainability. Gelatin is a protein-based animal derived biopolymer and has great potential for biomedical applications due to its biocompatibility, biodegradability and presence of cell adhesion RGD domains. As gelatin is produced by acid or base hydrolysis of collagen along with thermal treatments, gelatin loses most of its physical and covalent crosslinking after production. Due to this, it could only form a weak hydrogel below sol-gel temperature (20-25^{O}C) with limited elastomeric properties [1, 2]. To enhance the stretchability of gelatin hydrogels, supramolecular interactions need to be introduced in gelatin hydrogel. In this dissertation, a novel method for hydrogel fabrication was developed and optimized to enhance the stretchability of gelatin via supramolecular interactions. During elastomeric film fabrication, gelatin was exposed to water-tetrahydrofuran (water-THF) co-solvent system which caused structural transformation and has resulted in an elastomeric micro-porous film with a Young's modulus, ultimate tensile strength and strain at break of 78.71 ± 0.48 kPa, 516.7 ± 106.3 kPa and 1269±80. %, respectively. Structural transformation caused by co-solvent system involved transition from an aggregated state in water to spherical shaped coacervates at 43.8% THF with >90% transmittance in solution state. Further fusion of small spherical coacervates to form larger spherical coacervates took place with further increase in THF to 60%. This could have been caused by presence of multi droplet phase in water-THF co-solvent systems, which has caused fusion of smaller coacervates to form larger coacervates with increase in THF proportion.

Further increase in THF proportion to 80% have resulted in formation of an elastomeric micro-porous film. To remove the solvent, the film was dried in vacuum and rehydrated again at room temperature. The film underwent swelling till ~90% water content and started breaking into pieces due to loss of integrity. To enhance the stability elastomeric film, an external supramolecular crosslinking of ureidopyrimidinone (Upy) was introduced in gelatin. Upy is a hydrophobic self-assembled moiety developed by Meijer, which can undergo strong dimerization via quadrupole hydrogen bonding [3]. Upy was synthesized and substituted in gelatin via two step methods. Substitution was carried out in two steps with one step involving substitution of Upy to amino groups and the second step involves the addition of catalyst for Upy substitution at hydroxyl groups. To achieve different degrees of Upy substitution, Upy feed in the reaction was varied with 0.10, 0.30 and 0.50 g/2g gelatin to synthesize GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50). In situ co-solvent optimization studies have shown that the transmittance at 600 nm increases with increase in THF proportion as contrary to the gelatin alone without Upy substitution which had no significant change in transmittance till 43.8% THF. This could be due to decrease in hydrophobic aggregation due to solubility of Upy in THF as per earlier studies [4]. All Upy substituted gelatin derivatives were studied for their structural transformation at 50% THF. At 50% THF with 24 hours of incubation at 37 ^{o}C, some dissolved polymer fraction of gelatin, GEUPYII (0.10) and GEUPYII (0.30) sedimented at the bottom and the rest remained in supernatant. However, GEUPYII (0.50) remained as stable solution after incubation. From the SEM analysis of sedimented film and supernatant, it was observed that in case of gelatin the sedimented coacervates formed a continuous porous film due to aggregation of coacervates, as gelatin is not compatible with THF. In case of GEUPYII (0.10), the sedimented film was not continuous, whereas in GEUPYII (0.30), the coacervates present in sedimented film had fibrous and spherical structures and did not fuse to form a film. In case of GEUPUYII (0.50), no significant sedimentation was observed but only spherical coacervates in the solution state. Presence of spherical

aggregates in GEUPYII (0.50) could be due to the presence of stable moving droplet phase (MDP)/ coacervates formed during phase inversion at 50% THF. Due to higher Upy substitution in GEUPYII (0.50), the interactions between the solute and the solvent has increased, which may had prevented the coalescence of coacervates at 50% THF [5]. However, further increase in THF proportion had caused fusion of coacervates to form an elastomeric film similar to gelatin. This could be due to coalescence of coacervates during coarsening which took place through non-elastic collision of coacervates in liquid phase [5]. GEUPYII (0.50) was chosen to study the fusion of coacervates and elastomeric film fabrication, since it had the highest Upy substitution required to form a supramolecular network. In GEUPYII (0.50), adjustment of THF proportion from 50% to 52.5%, 60% and 70% THF and instantaneously freezing of samples after 30 seconds mixing followed by freeze-drying for SEM analysis had shown the formation of large micro-sized spherical coacervates, probably caused by fusion of smaller aggregates in case of 60% and 70% THF. Also, the proportion of large micro-sized coacervates was higher in 70% THF in comparison to 60% THF, demonstrated the effect of THF on the fusion of aggregates.

GEUPYII (0.50) elastomeric film fabrication was carried out at 80% THF, similar to gelatin. The elastomeric film of GEUPYII (0.50) 80% THF had 10.13 ± 1.3 kPa Young's modulus with 207.96 ± 4.8 kPa ultimate tensile strength and 1405.9 ± 47.9 % strain at break. The GEUPYII (0.50) 80% THF film had a higher strain at break but lower Young's modulus (10.13 ± 1.3 kPa vs 78 .71 ± 0.48 kPa) and ultimate tensile strength (207.96 kPa vs 516.7 ± 106.3 kPa) as compared to gelatin elastomeric film. GEUPYII (0.50) 80% THF film was air dried in vacuum and was rehydrated with water. Water adsorption reached to ~70% within 2 minutes and remained constant when observed for 48 hours and was stable. Rehydrated air-dried films of GEUPYII (0.50) had 318.7 ± 44.4 %, strain at break with 27.35 ± 2.7 kPa, Young's modulus and 88.57 ± 50.3 kPa, ultimate tensile strength. Rehydration of air-dried films had lower strain at break (318.7 ± 44.4 %, vs 1405.9 ± 47.9 %), lower ultimate tensile strength

(88.57 ± 50.3 kPa vs 207.96 ± 4.8 kPa) and higher Young's modulus (27.35 ± 2.7 kPa vs 10.13 ± 1.3 kPa) as compared to GEUPYII (0.50) 80% THF films. This could be due to decrease in dissipative supramolecular networks and increase in hydrophobic interactions due to replacement of water-THF co-solvent with water which had decreased the stretchability and increased the stiffness of the hydrogel, respectively. The abovementioned study has established a novel method of elastomeric film fabrication for gelatin based hydrogels and Upy crosslinking reinforced gelatin has been fabricated into a stretchable hydrogel with higher stretchability in comparison to native gelatin based hydrogel and covalently cross-linked gelatin based hydrogels.

Acknowledgements

I am thankful to all the people who have supported me throughout my research work and study.

I am really thankful to School of Material Science and Engineering, Nanyang Technological University (NTU), Singapore and Ministry of Education (MOE), Singapore for the technical, infrastructure, administrative and financial support throughout my PhD journey.

I would like to express my gratitude to my supervisor Associate Professor Tan Lay Poh for her guidance throughout my project work. She is one of the most generous and intellectual person I have known. She has always motivated me and have shown unconditional support during my PhD journey. Her valuable advice at every step of my project work has made me learn and explore new aspects of research. I would like to thank Professor Lam Yeng Ming (Director of Facility for Analysis Characterization Testing and Simulation, Nanyang Technological University, Singapore) for the structural characterization studies in my project work. My sincere thanks to Associate Professor Ali Miserez in providing access to the chemical characterization facilities of his lab required for my project work.

I would like to thank Dr. Lee Bae Hoon, Dr. Sk Md Moniruzzaman, Dr. Deepan kumar and Dr. Ajay Sanjay Tijore in assisting me during my project work. I am grateful to my labmates Dr. Chen Huizhi and Prativa Das for the discussions and support. An extended gratitude to Dickescheid Andreas for the fruitful discussions and assistance.

Last but not the least, I would like to thank my family: my parents, my parents-in-law, my friends (Jitender Giroh, Bharat Mural and Deepak Nehra) for supporting me spiritually and my life in general during my project work. And special thanks to my wife, Geetika Sahni for being there for me as a support.

Table of contents

Figure Captions

Table captions

Abbreviations

6 MC	6-methylcytosine
$CDCl_3$	Deuterated chloroform
DBTDL	Dibutyltin dilaureate
DMSO	Dimethyl sulfoxide
DMSO-d6	Deuterated dimethyl sulfoxide
FTIR	Fourier Transform Infrared Spectroscopy
Gel-Ada	1-adamantyl isothiocyanate substituted gelatin
Gel-Phe	Phenyl isothiocyanate substituted gelatin
HA	Hyaluronic acid
HDI	Hexamethylene diisocyanate
IR	Infrared radiation
MDP	Moving droplet phase
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NMP	N-methyl-2-pyrrolidone
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
PAA	Poly (acrylic acid)
PAM	Polyacrylamide
PBB	Protein based biopolymer
PBEH	Protein based elastomeric hydrogels
PC	Poly (citrate)
PCL	Poly (caprolactone)
PCL-diol	Polycaprolactone-diol
PDMA	Polydimethylacrylamide
PEG	Poly (ethylene glycol)
PEG-Upy	Ureidopyrimidinone substituted poly (ethylene glycol)
PEI	Poly (ethylenimine)
PNIPAM	Poly (N-isopropylacrylamide)
PVA	Poly (vinyl alcohol)

PVP	Poly (vinylpyrrolidone)
SEM	Scanning Electron Microscopy
SX	Solvent exchange
THF	Tetrahydrofuran
Upy	Ureidopyrimidinone
UV	Ultra-violet radiation
WIHP	Water insoluble hydrophilic polymers
WSHP	Water soluble hydrophilic polymers

Chapter 1

Introduction

This chapter presents the background (section 1.1) of the elastomeric hydrogels and the hypothesis (section 1.2). Protein based elastomeric hydrogels with supramolecular interactions are subsequently introduced with role of supramolecular interactions to strengthen the weak hydrogels, which is presented in scope and objectives (section 1.3). Finally, the significance and novelty of the research with overview are presented (section 1.4 and 1.5).

1.1 Background

Protein based elastomeric hydrogels (PBEH) are gaining popularity for their application in biomedical due to their self-assembly behaviour, biocompatibility, bioactivity and tunable porous microstructure [5]. Proteins and polypeptides for the fabrication of PBEH are derived either from the natural sources (animal/ insects) or via recombinant bacterial expression, which can be further crosslinked either physically or covalently [6, 7]. Limited number of natural proteins or polypeptides based elastomeric hydrogels have been reported in the literature, which includes elastin, resilin and flagelliform spider silk [7-10]. These proteins and polypeptides were synthesized using recombinant bacterial strain and were developed into elastomeric hydrogels. However, production of recombinant protein for the fabrication of PBEM is expensive and difficult to scale up for large scale production. Apart from these proteins, there are other animal derived protein-based biopolymers like gelatin, keratin, fibrin, collagen etc have limited elasticity but have a great potential due to biocompatibility, biodegradability, bioactivity and sustainability. Despite of such potential, their application has been limited by various limitation including batch to batch variation, stability, limited physical crosslinking and low mechanical properties [11, 12]. Various physical, enzymatic and chemical crosslinking methods have been developed by researchers to improve stability and mechanical properties [13]. However, enzymatic and chemical crosslinking methods are unable to enhance the elasticity of the hydrogels due to formation of tough crosslinked network which would undergo limited physical deformation to be recovered.

Physical crosslinking in polymeric systems has been strengthened by researchers either by blending with other polymers or by substitution of self-assembled functional groups [14]. Introduction of physical crosslinking has been observed to form a flexible crosslinked network, which can undergo reversible physical deformation to form an elastomeric hydrogel [15]. Self-

assembled molecules used for substitution involves host-guest complexes, inclusion complexes and self-dimerized moieties and have been exploited to form elastomeric hydrogels from synthetic polymers [16-18]. Substitution of given self-assembled molecules in natural biopolymers have not been much explored by the researchers and requires extensive study to exploit the potential of biopolymer limited by the abovementioned issues.

1.2 Hypothesis

Gelatin is an animal derived protein-based biopolymer, capable of forming a weak, brittle, physical cross-linked hydrogel below sol-gel transition temperature with limited stretchability. Thus, we hypothesize that substitution of ureidopyrimidinone on gelatin biopolymer and its hydrogel network fabrication through restructuring by co-solvent method would introduce hydrogen bonding and hydrophobic interactions, which would strengthen the physical crosslinking to form an elastomeric hydrogel. Introduction of hydrogen bonding through Upy dimerization and hydrophobic interactions would transform a weak hydrogel into an elastomeric hydrogel with potential biomedical applications.

1.3 Research objectives and scope

The objective of this thesis is fabrication of gelatin based stretchable hydrogel by introducing supramolecular interactions via substitution of ureidopyrimidinone on gelatin biopolymer. Ureidopyrimidinone has the potential to undergo self-dimerization due to intermolecular hydrogen bonding and introduce hydrophobic interaction in the host polymer, when exposed to aqueous environment. From these studies, hydrogel fabrication via solvophobic interaction and role of co-solvent in restructuring of the gelation biopolymer to form a stretchable hydrogel has been established.

The objectives and scopes of the research are specified as:

1. To fabricate gelatin based stretchable elastomer via co-solvent method

- Investigation of gelatin solubility in co-solvent systems
- Investigation of co-solvent system induced structural and chemical transformation
- Fabrication of gelatin based stretchable elastomer via co-solvent system
- Exploration of elastomeric film fabrication mechanism
- Studies to investigate the mechanical and physical properties of elastomeric films

2. To synthesize and characterize self-assembled moiety ureidopyrimidinone and its substitution in gelatin biopolymer

- Synthesis of ureidopyrimidinone and its chemical characterization for structural analysis
- Synthesis of ureidopyrimidinone substituted gelatin derivatives (GEUPYII) via two step method with different degrees of substitution by varying reactants composition
- Investigation of ureidopyrimidinone substitution in gelatin biopolymer via chemical characterization

3. To fabricate elastomeric hydrogel of Upy substituted gelatin derivatives

- Investigation of Upy substituted gelatin derivatives solubility in co-solvent systems
- Investigation of co-solvent system induced structural transformation in Upy substituted gelatin derivatives
- Fabrication of Upy substituted gelatin derivatives based stretchable elastomer via co-solvent system

- Structural and chemical analysis of elastomeric film fabricated by co-solvent system
- Solvent exchange of elastomeric films and investigation of physical and mechanical properties

1.4 Significance and novelty

This dissertation demonstrates gelatin biopolymer based elastomeric gels can be fabricated by co-solvent system which introduces solvophobic interactions to enhance the stretchability of gelatin biopolymer. However, elastomeric gel of gelatin loses its elastomeric properties and stability after solvent exchange. To enhance the stability, gelatin biopolymer was modified with ureidopyrimidinone to introduce supramolecular crosslinking via hydrogen bonding and hydrophobic interaction. Hydrophobically modified polymers often undergoes intra-chain crosslinking and forms micellar structures, preventing network formation for hydrogel fabrication. Hydrogel fabrication via co-solvent method was developed and have assisted in decreasing intramolecular crosslinking caused by spontaneous hydrophobic aggregation. Co-solvent system has been shown to prevent intramolecular interaction and assist in formation of intermolecular interactions for hydrogel fabrication. The novelty of this study is fabrication of a stable elastomeric hydrogel via novel co-solvent method, which has enhanced the stability as well as stretchability of gelatin biopolymer by many folds by imparting a flexible supramolecular cross-linked energy dissipative network.

1.5 Dissertation overview

This thesis consists of the following seven chapters:

Chapter 1 briefly presents the background of the study along with an introduction of research objectives/scopes as well as significance/novelty of this project, and finally outlines the overview of the thesis.

Chapter 2 covers a detailed literature review of relevant topics on hydrogel, gelatin and gelatin based stretchable hydrogels fabricated via covalent and supramolecular crosslinking, ureidopyrimidinone and ureidopyrimidinone substituted polymers and their properties, hydrogel fabrication methods for supramolecular cross-linked polymers

Chapter 3 introduces the main materials and experimental methods, or techniques involved in this dissertation.

Chapter 4 demonstrated the structural and chemical transformation by co-solvent systems on gelatin biopolymer, followed by fabrication of gelatin-based elastomer and its structural, physical and mechanical properties.

Chapter 5 demonstrates the synthesis and chemical characterization of ureidopyrimidinone and ureidopyrimidinone substituted gelatin derivatives

Chapter 6 demonstrates the solubility and structural analysis of ureidopyrimidinone substituted gelatin derivatives followed by fabrication of elastomeric hydrogel with its structural, chemical and mechanical characterization.

Chapter 7 briefly gives some recommendations for future perspectives

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Chapter 2

Literature Review

This chapter 2 starts with introduction to hydrogels and their mechanism of formation, followed by introduction to gelatin with its structural and chemical properties in section 2.2. Section 2.3 introduces to the gelatin based stretchable hydrogels and various strategies to fabricate them. Ureidopyrimidinone and its employment in various hydrophilic polymers is discussed in section 2.4. Section 2.5 described the hydrogel fabrication strategy adopted for supramolecular hydrogels

2.1 Introduction to hydrogels

Hydrogels constitute a three-dimensional hydrophilic polymer network, which can hold water in its swollen state as shown in Figure 2.1 [1]. Hydrogels can be classified into synthetic, natural and hybrid hydrogels based on the source of polymer network. These polymer networks are held together by chemical, physical or both crosslinking in a hydrogel. Hydrogel properties are controlled by the crosslinking, chemical and physical structure of polymer network. Water sorption, mechanical strength, rheology and stability are among the most common properties studied for typical hydrogels. Water sorption in a polymer network is a result of various interaction including capillary, osmotic and hydration forces and are counter balanced by the cross-linked polymer network. The hydrogel reaches its terminal swelled state at equilibrium between these forces, which also defines the mechanical strength, diffusion characteristics and internal transport [2]. Hydrogels are among the most common biomaterials due to substantial water content, imitating *in vivo* microenvironment of animal tissues. Nature derived biopolymers have been fabricated as hydrogels by researchers for various biomedical applications including synthetic peptides, plant-derived biopolymers (alginate, dextran and cellulose) and animal-derived biopolymers (chitosan, gelatin, fibrin and silk). For biomedical applications like tissue engineering, drug delivery vehicles and diagnostic platforms, where biocompatibility, biodegradability and cellular toxicity are major concerns, the employment of animal derived protein-based biopolymer is a viable option due to their animal origin especially gelatin biopolymer. Gelatin is one of the most suitable protein-based biopolymers for hydrogel application in biomedical industry due to sustainability, biocompatibility, cell binding domains and biodegradability. It has been incorporated by the researchers in synthetic and other natural hydrogels to enhance their cell supportability [3-5].

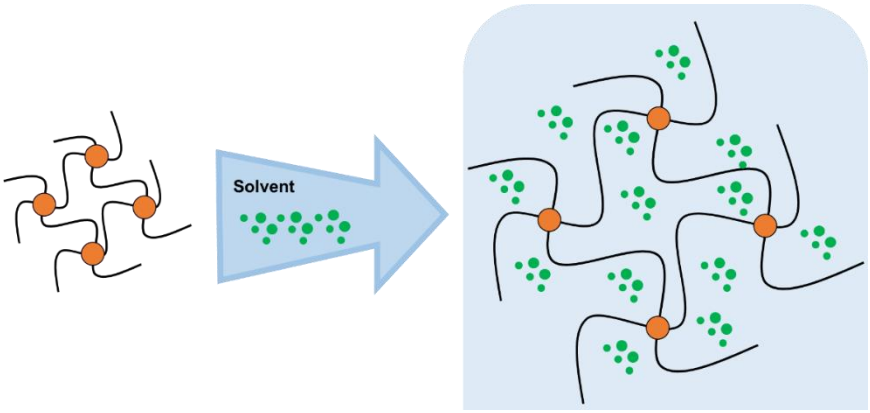

Figure 2.1. Schematics displaying the swelling of a cross-linked polymer network to form a hydrogel

2.2 Gelatin

Gelatin is a biopolymer derived majorly from bovine hides (29.4%), pig skin (46%) and bones of pig and cattle (23.1%) [6]. Skin and bones are usually obtained from slaughter houses and meat processing industries. The global gelatin market size was 412.7 kilo tons (2015) and is growing with growth in meat industry [7]. It has been employed for various applications including food and beverages, pharmaceuticals, nutraceuticals, photography and personal care. It is produced through acid (gelatin A) or alkaline hydrolysis (gelatin B) of collagen present in the sources, mentioned above. The chemical composition or amino acid composition of gelatin (shown in Table 2.1) is similar to collagen with a few changes caused by the manufacturing process. Gelatin B has higher glutamic acid and aspartic acid content due to alkaline hydrolysis of glutamine and asparagine as shown in Table 2.1. Due to this conversion the isoelectric pH of both gelatin differs with 8-9 pH for gelatin A and 4.8-5.5 pH for gelatin B [8, 9]. It forms a soft gel upon cooling due to renaturation of triple helixes as shown in Figure 2.2.

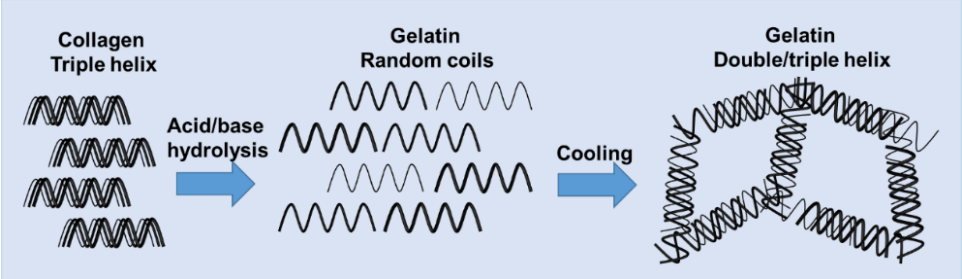

Figure 2.2 Schematics of collagen, gelatin and renaturation of gelatin random coils upon cooling

Table 2.1 Amino acid composition of collagen and four different types of composition (% amino acids) [13]

Amino acid	Type I Collagen (bovine)	Type A gelatin	Type B gelatin	Cold water fish gelatin	Warm water fish gelatin
Alanine	11.4	11.2	11.7	11.2	12.3
Arginine	5.1	4.9	4.8	4.9	4.7
Asparagine	1.6	1.6	4.6	4.8	4.8
Aspartic acid	2.9	2.9	0	0	0
Glutamine	4.8	4.8	7.2	7.2	6.9
Glutamic acid	2.5	2.5	0	0	0
Glycine	33.2	33	33.5	34.7	34.7
Histidine	0.4	0.4	0.4	1.1	0.6
4-4-hydroxyproline	10.4	9.1	9.3	6	7.9
Hydroxylysine	0.5	0.6	0.4	0.5	0.8
Isoleucine	1.1	1	1.1	1.1	0.8
Leucine	2.4	2.4	2.4	2.1	2.3
Lysine	2.8	2.7	2.8	2.8	2.5
Methionine	0.6	0.4	0.4	0.3	0.9
Phenylalanine	1.3	1.4	1.4	1.3	1.3
Proline	11.5	13.2	12.4	9.6	11.9
Serine	3.5	3.5	3.3	6.3	3.5
Threonine	1.7	1.8	1.8	2.4	2.4
Tyrosine	0.4	0.3	0.1	0.9	0.2
Valine	2.2	2.6	2.2	1.8	1.5

2.2.1 Structure and chemical composition

Collagen is the most abundant fibrous protein of animal extracellular matrix and its acid/based hydrolysis leads to formation of gelatin. Collagen monomer called tropocollagen is a triple helix made up of three α-chains shown in Figure 2.3. Tropocollagen is 300nm in length and 1.5nm is diameter with a molecular weight of 300000 Da [10]. The basic amino acid sequence of collagen is Gly-X-Y, where X is usually proline and Y is 4-hydroxyproline. It implies that glycine constitutes ~33% of total amino acids as shown in Table 2.1, whereas proline and 4-hydroxyproline constitutes ~11% and ~9%, respectively. The basic amino acid sequence and composition of gelatin remains similar to collagen after acid/base hydrolysis with a few changes which involves loss of covalent/non-covalent cross-links and deamination of glutamine and asparagine in basic hydrolysis. During manufacturing process of gelatin, the triple helical structure of collagen unfolds into a mixture of three α-chains with random coil structure (random coils). These random coils have the potential to undergo renaturation to form triple helixes and triple helixes bundles below sol-gel temperature due to hydrogen bonding. The random coils, triplex helixes and triples helixes bundles are shown in Figure 2.4.

4-Hydroxyproline of collagen is not a natural amino acid and its formation takes place via hydroxylation of proline residue by prolyl 4-hydroxylase during post translational modification of collagen in endoplasmic reticulum [11]. Presence of hydroxyl group in 4-hydroxyproline has been reported to assist in stabilizing the triple helix structure of collagen in physiological conditions [11]. The stability provided by the 4-hydroxyproline is due to stereo-electronic effect [12, 13]. Raines and co-workers have demonstrated that the inductive effect of hydroxyl group along with stereochemistry of the Cγ substituent have a significant effect on stability of structure. Replacement of 4-hydroxyprolines with (2S, 4R)-4-fluoroproline (Flp) in Glycine-proline-hydroxyproline tripeptide has resulted in formation of hyperstable triple helix with 91^{o}C

melting temperature in comparison to triple helixes by peptides with in Glycine-proline-hydroxyproline tripeptide. The hyperstability of triple helixes has been reported due to higher electronegativity of fluorine than oxygen [14]. It has been reported that trans conformation of the peptide bond and electron withdrawing group at 4(R) position in proline contribute to the stability of collagen structure [12]. Similar to synthetic peptides with tripeptide repeat, presence of hydroxyproline in tripeptide of gelatin also plays a vital role in renaturation of random coils into triple helixes. Apart from this, the hydroxyl group have been found to form inter chain γ and intra chain δ bridges demonstrated in the studies reported by Berman and co-workers via X-ray crystallography as shown in Figure 2.5 [15].

Beside 4-hydroxyproline, polarity and charge over the α-chains also determine the folding of chains into a triple helix in collagen. The collagen α-chains have polar and non-polar regions. The tripeptide Gly-Pro-X, X is a non-polar amino acid, most commonly 4-hydroxyproline forms the non-polar regions of the α-chains [16]. These non-polar tripeptides are interspersed by polar region, which lacks proline or 4-hydroxyproline [17]. Altogether, charged, polar and non-polar regions make the structure and properties of collagen unique. Insolubility of collagen in water is due to the hydrophobicity attributed by non-polar regions and covalent crosslinks whereas gelatin can be easily dissolved in water under similar conditions due to the loss of crosslinks during hydrolysis, charge and hydrophilic segments [18]. Gelatin forms a kind of colloidal solution and has been regarded as multifunctional hydrocolloid by definition [19].

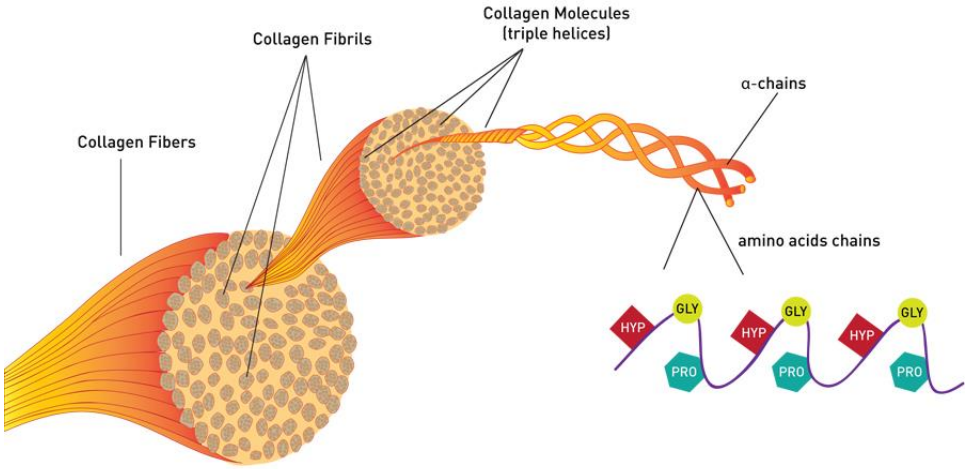

Figure 2.3 Structure of collagen fibrils [20]

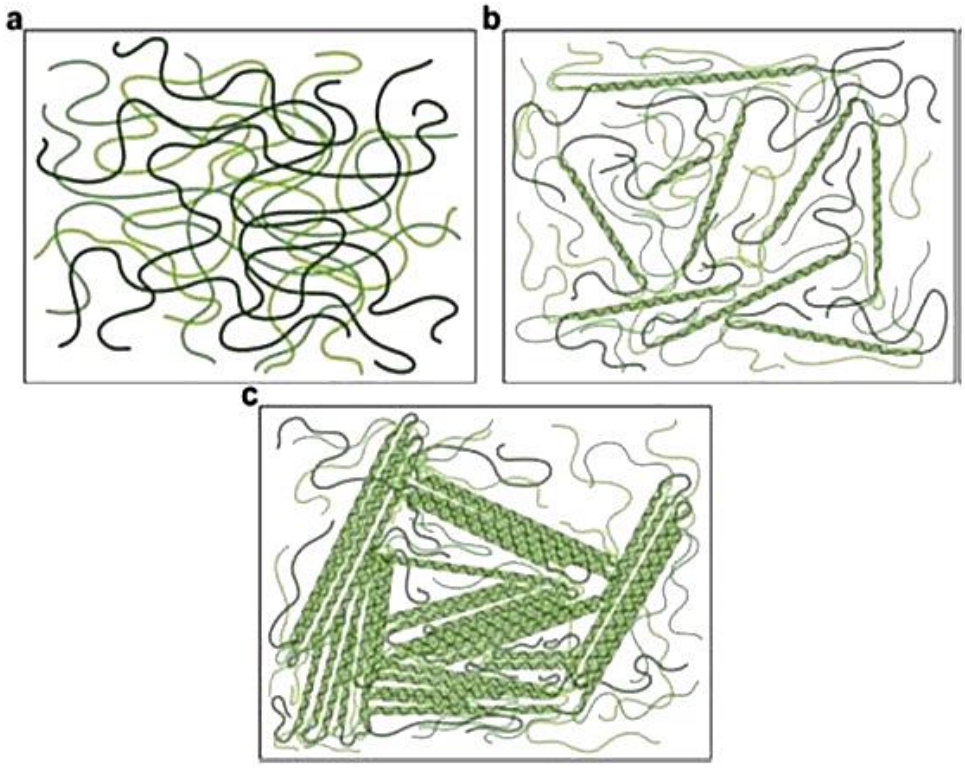

Figure 2.4 Structure of gelatin a) random coils; b) triple helixes and random coils; c) triple helixes, triple helix bundles and random coils [21]

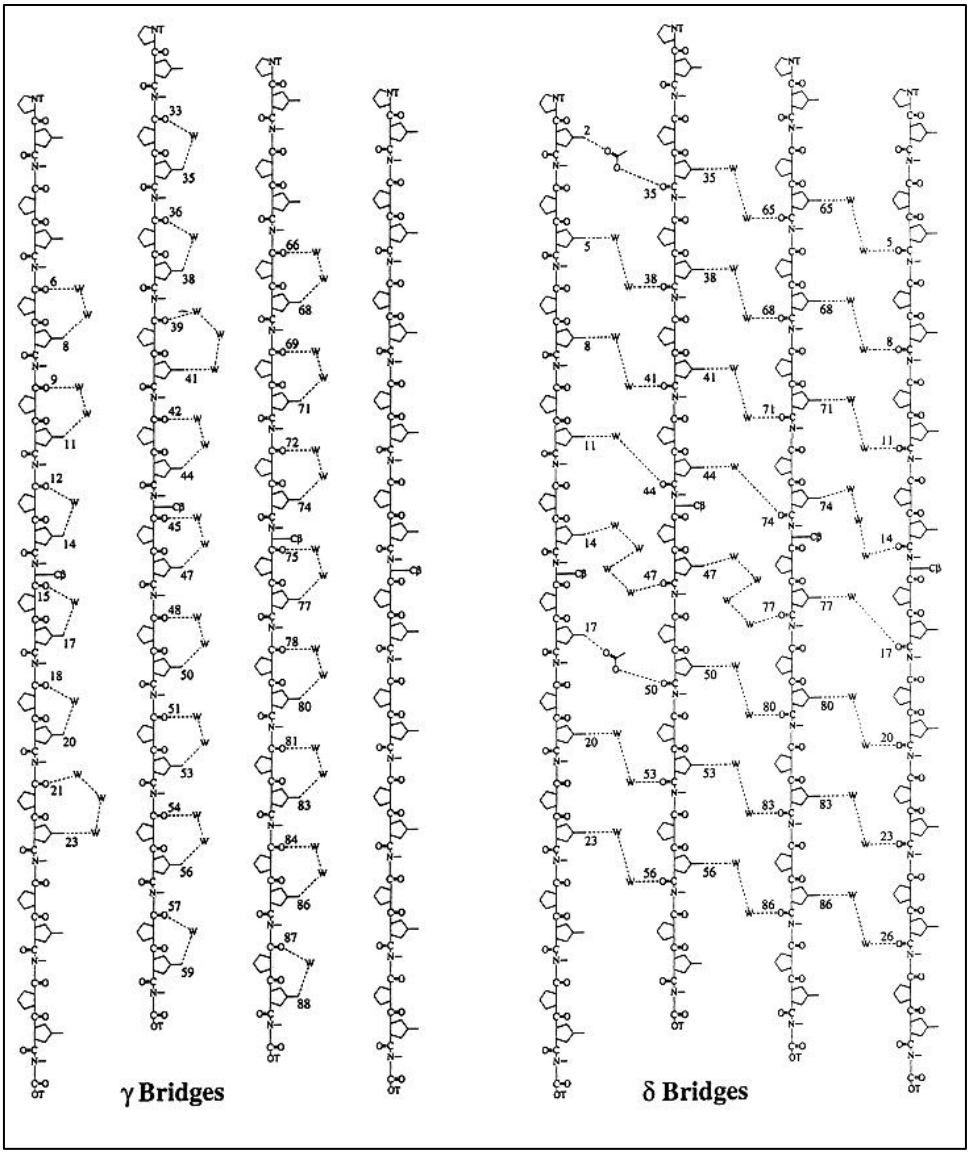

Figure 2.5 Inter and intra chain bridges by 4-hydroxyproline hydroxyl group and carbonyl group [15]

2.2.2 Physical properties of gelatin

Gelatin can easily be dissolved in warm water and forms a thermos-sensitive hydrogel below sol gel transition temperature (20-25^{o}C) [22]. The sol-gel transition temperature varies with concentration and bloom number. Zhao et al have demonstrated the change in gelation/sol-gel transition temperature with concentration. It was shown that the gelation temperature increases from 21-28.5^{o}C, when the concentration increased from 5% to 20% [23]. It exhibits as low viscosity liquid above gelation temperature. It is known to form random coils in warm water, which would undergo a transition from coil to helix formation with decrease in temperature. The gelation of gelatin biopolymer has been studied during heating or cooling conditions. It has been found that the triple helix begins to form due to inter-chain hydrogen bonding with increase in cooling below melting point as shown in Figure 2.6. Multiple triple helixes are joined to many segments of random coils. These triple helixes will reorganize multiple times to achieve the minimum free energy and could take several weeks in reaching equilibrium. The gelation time varies with concentration due to the formation of large number of coils to helix transformation [24]. During cooling of gelatin solution, the viscosity of gelatin solution increase, which also demonstrates the formation of physical crosslinking in gelatin biopolymer. The gelatin manufactured by various sources and processes would yield gelatin with a wide range of molecular weight. To classify them Oscar T. Bloom developed a bloom-test to classify the gelatin based on melting and gelling point [25]. Higher bloom number represent higher melting and boiling point and fast gelation (shown in Table 2.2).

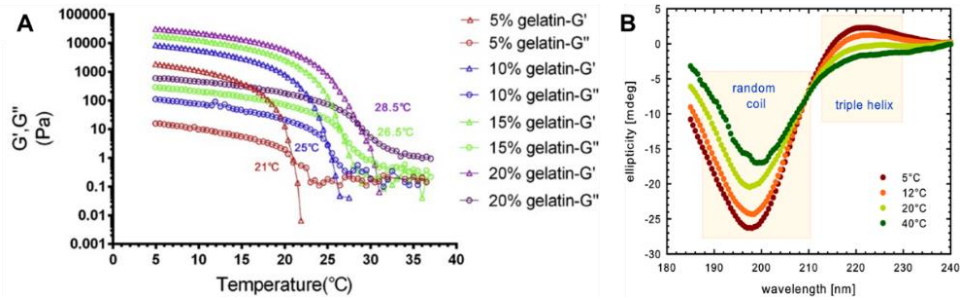

Figure 2.6. A) Loss and storage modulus of gelatin at different temperature and concentrations [23]; B) Circular dichroism spectrum of gelatin ate different temperature demonstrating the formation of triple helix with decrease in temperature [26].

Table 2.2. Classification of gelatin by bloom number

Sno.	Bloom type	Bloom number	Average molecular mass
1	Low	50-125	20-25k
2	Medium	175-225	40-50k
3	High	225-325	50-100k

2.3 Gelatin based stretchable hydrogels

Gelatin form a soft hydrogel below sol-gel temperature and have limited stretchability due to physical crosslinking. Physical crosslinking involved triple or double helix network formed by random coils. Pure gelatin has been reported to have 192% strain (ε) at break. Due to its instability under physiological conditions, it has been cross-linked with many crosslinking agents including chemical and enzymatic agents. In a study reported by Adriana and co-workers, the strain at break decreases from 192 ± 27% to 16 ± 4 % and 14 ± 2% with increase in crosslinking and modulus increases from 2.9 ± 0.4 M Pa of pure

gelatin to 10.3 ± 0.7 M Pa and 18 ± 3 M Pa for genipin and glutaraldehyde cross-linked gelatin, respectively [27, 28]. This demonstrates that the covalent crosslinking via chemical or enzymatic crosslinking has decreased the stretchability but enhanced the Young's modulus of gelatin hydrogel and stability under physiological conditions. For biomedical application especially for application like wound healing or tissue engineering, the modulus of hydrogel and stretchability should be comparable to skin and tissues. The chemical crosslinking would increase the young's modulus which is way higher in comparison to natural tissues. Figure 2.7 demonstrates the young's modulus for the natural tissues. Both enzymatic and chemical crosslinking facilitates the stability and a static 3D polymer network for hydrogel fabrication. This static and stable interface prevent the sliding of protein or polymer chains and would result in a decrease in stretchability of the resultant hydrogel. To enhance the stretchability of gelatin based hydrogels, various methods as outlined in the following sub-chapters have been attempted.

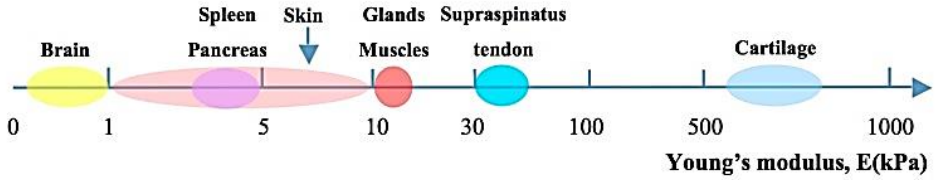

Figure 2.7 Schematics demonstrating the young's modulus of various human tissues [29]

2.3.1 Blending

Gelatin has been blended with other stretchable hydrogels to develop a gelatin based stretchable hydrogels. Feng et al. had blended polycitrate (PC) with gelatin (G) hydrogels in different proportion (PC: G, 1:0.25; 1:0.5; 1:1) to fabricate stretchable hydrogels. The strain at break was ~1300%, ~900% and ~300% for 1:0.25, 1:0.50 and 1:1 (PC: G), respectively (as shown in Figure 2.8) [30]. The strain at break decreases with increase in gelatin proportion. This

demonstrates that simple blending of gelatin with other polymers may not be able to yield the best combination of mechanical properties.

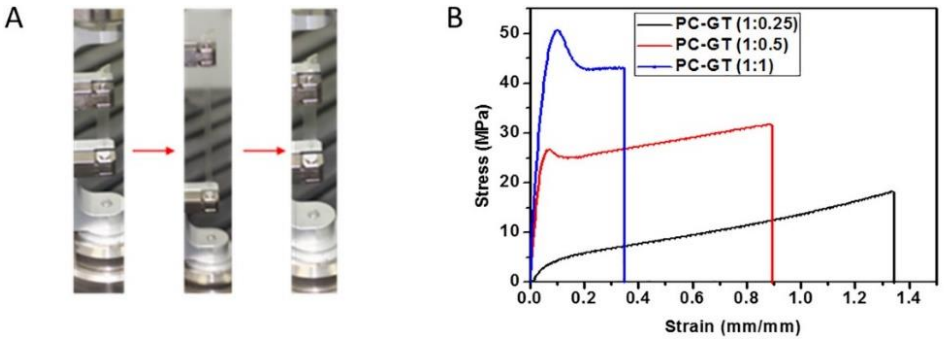

Figure 2.8 (A) Optical images showing the tensile and recovery process; (B) Stress-strain curves of polycitrate-gelatin hydrogel [30]

2.3.2 Double network hydrogels

Double network hydrogels have been fabricated with synthetic hydrogels to fabricate gelatin based stretchable hydrogels. Zheng and co-workers have reported double network hydrogels formed by polymerization of acrylamide in the presence of gelatin. In this study, the strain at break (ε_f) for double network hydrogel was 40.69 mm mm^{-1} with 0.268 MPa, ultimate tensile strength. The stretchability of double network hydrogel was higher in comparison to acrylamide hydrogel (shown in Figure 2.9) and also have self-healing properties due to renaturation of triple helixes [31]. However, the given method relies on the external network of synthetic polymer which is non-biodegradable.

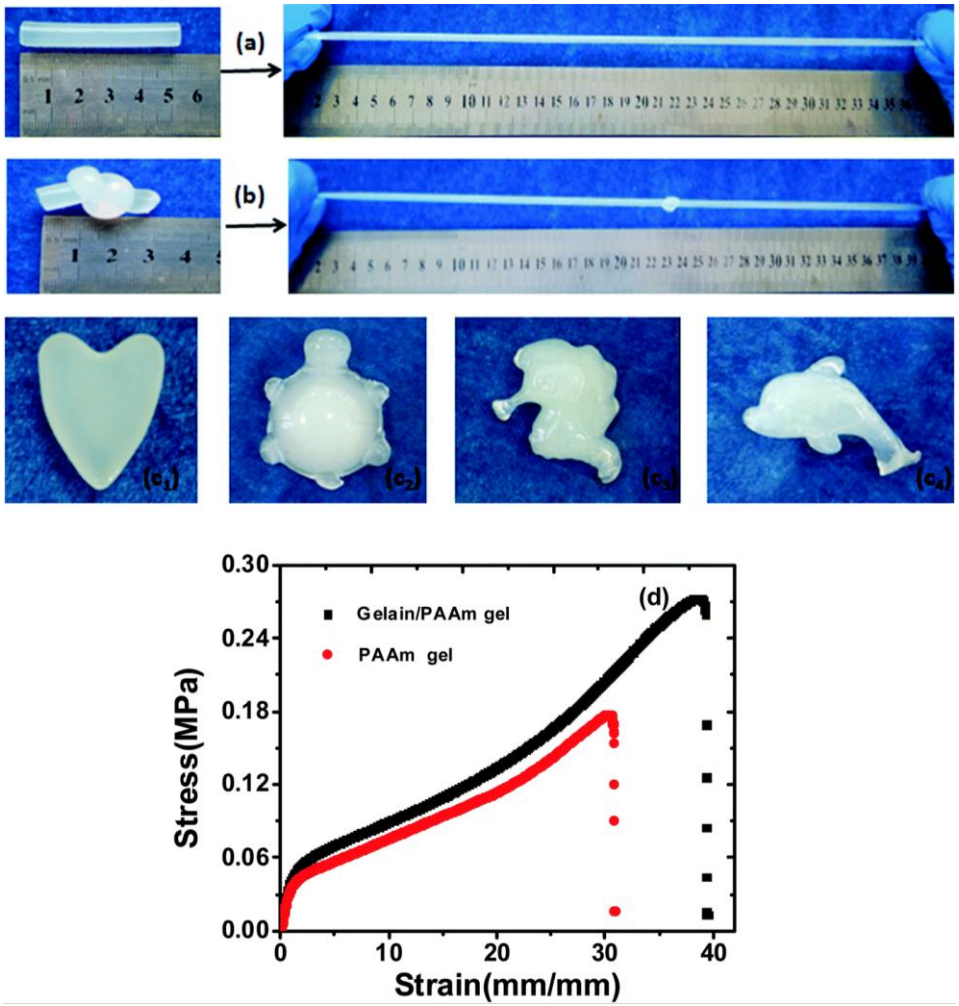

Figure 2.9 A) Elastomeric gelatin polyacrylamide gel in extended state; B) Knotting in gelatin polyacrylamide hydrogel; C) Hydrogel fabricated in various shapes; D)Stress-strain curve of gelatin and gelatin-polyacrylamide hydrogels [31]

2.3.3 Functionalization of gelatin to introduce secondary crosslinking

Substitution of a functional molecule over polymer chain is one of the common strategies used by researchers to introduce the crosslinks formed by the substituted molecules. Both covalent and non-covalent crosslinks have been introduced by the researchers to strengthen the gelatin hydrogels through

substitution. Substitution of methacrylate in gelatin hydrogels for UV crosslinking would be able to enhance the strain in the range of 50-150%, depending upon the number of crosslinks. Xu and co-workers have reported an increase in strain of methacrylate substituted gelatin hydrogel, when exposed to UV for ~20 seconds and it decreases to almost negligible strain if exposure is ~60 seconds as shown in Figure 2.10 [32]. Batch to batch variation of gelatin makes the optimization of UV curing method challenging and use of toxic photo-initiators makes it unsuitable.

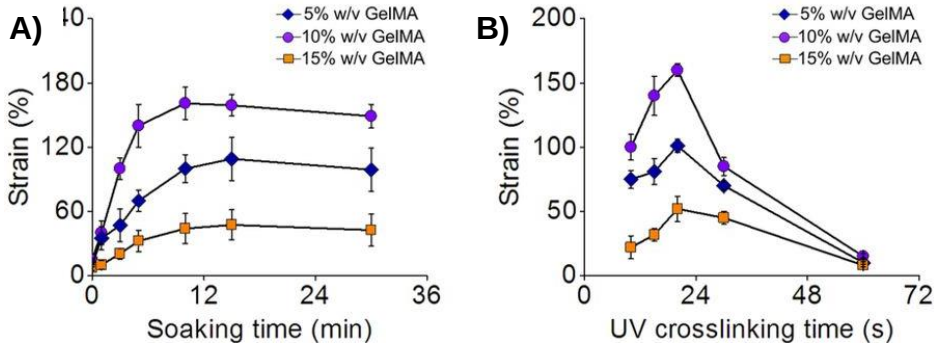

Figure 2.10 A) Failure strain of methacrylate substituted gelatin hydrogels when soaked in water for different interval of time (UV exposure - 20 seconds); B) Failure strain of methacrylate substituted gelatin hydrogels when exposed to UV for different interval of time [32]

Apart from covalent crosslinking, gelatin has been modified with functional moieties to introduce non-covalent crosslinking. Gelatin has been modified with hydrophobic groups to introduce hydrophobic interactions. Substitution of 1-adamantyl isothiocyanate and phenyl isothiocyanate in gelatin along with free unreacted molecules (adamantyl and phenyl derivatives) has enhanced the stretching to 34 and 42 times of their original length as shown in Figure 2.11. However, hydrogel failed to form in absence of unreacted molecules (left after the substitution reaction), which demonstrates deficiency of enough crosslinks formed by substituted functional groups to stabilize the hydrogel structure. Hydrophobic group substitution usually results in loss of hydrogel forming ability in aqueous medium due to non-directional interaction which cause

random aggregation and fails to form a network. In this abovementioned study, the hydrogel fabrication method used for fabrication was based on solvent exchange which involves the exchange of DMSO (reaction solvent) with water through dialysis [33].

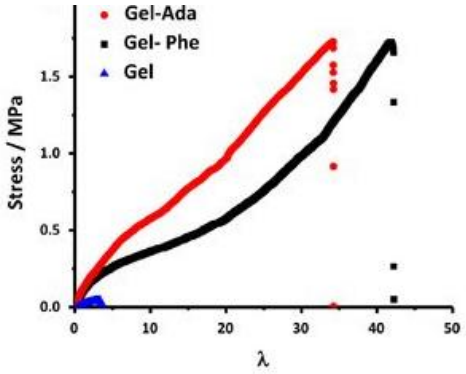

Figure 2.11 Stress-λ ((stretch ratio: stretched length/original length) of gelatin substituted with 1-adamantyl isothiocyanate (Gel-Ada) and Phenyl isothiocyanate (Gel-Phe) [33]

Gelatin has been physically cross-linked via UV cross-linked network of acrylated β-cyclodextrin (Form complex with aromatic amino acids) to achieve ~403%, strain at break (shown in Figure 2.12) [34]. Earlier reports of β-cyclodextrin and adamantane modified gelatin failed to form a hydrogel due to lack of crosslinking and requires the UV crosslinking of acrylate β-cyclodextrins [35]. Among non-covalent crosslinking in hydrogels, hydrogen bonding has not been investigated to a greater extent due to interference by water molecules. Gelatin has been modified with Ureidopyrimidinone (Upy) to introduce crosslinks formed by hydrogen bonding between Upy moieties. The hydrogel fabrication was attained via solvent casting in formic acid followed by rehydration due to insolubility issues of Upy modified gelatin. The strain at break at 75% swollen hydrogel and fully swollen hydrogel was 12.58% and 11.75%, respectively as shown in Figure 2.13 [27]. The stretchability is almost like gelatin methacrylate, which demonstrates the absence of energy dissipation network to make it stretchable. In another study of Upy substituted gelatin, ferric ion crosslinking in addition to Upy crosslinking has been reported to form

a stable self-healing hydrogel [36]. In earlier studies, Upy has been substituted in polymers which have solubility in organic solvents. However, its substitution in water soluble polymers has been studied by few researchers and require an extensive study to explore its potential in water soluble polymers.

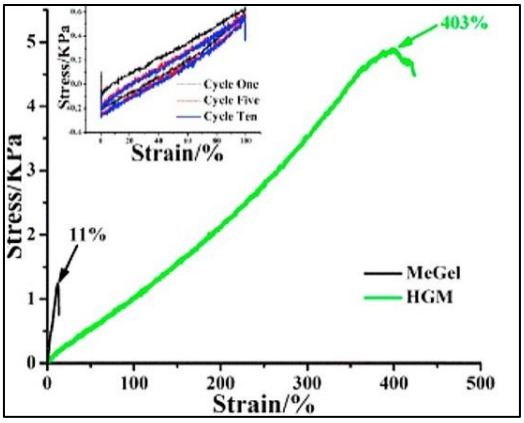

Figure 2.12 Stress strain curve of methacrylate gelatin (MeGel) and host guest macromere (HGM) hydrogels [34]

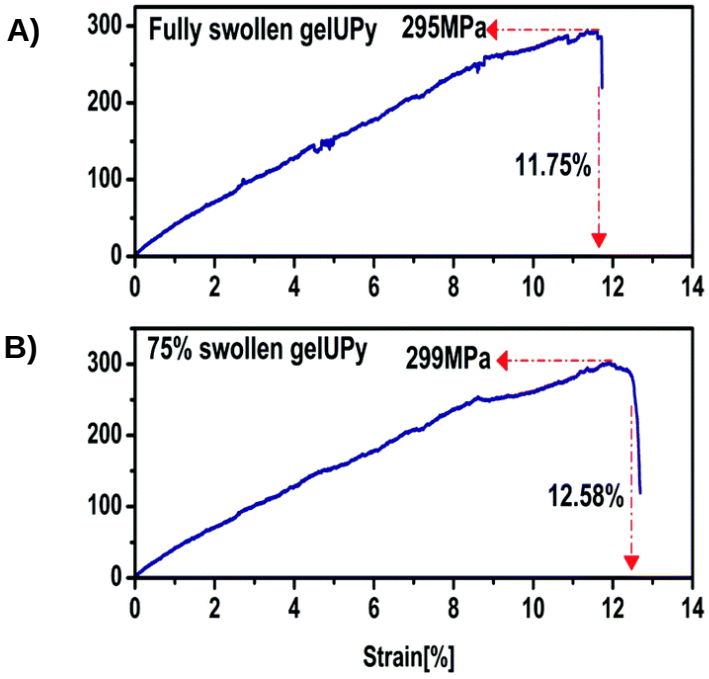

Figure 2.13 Stress strain curve of Upy substituted gelatin hydrogel with fully swollen and 75% swellable hydrogel [27]

2.4 Ureidopyrimidinone substituted hydrogels

2.4.1 Ureidopyrimidinone and its properties

Ureidopyrimidinone is a pyrimidine derivative with strong dimerization potential developed by Meijer group [37]. It undergoes reversible dimerization via quadrupole hydrogen bonds shown in Figure 2.13. It is hydrophobic and possess strong dimerization constant of 10^6 M^{-1} in chloroform [38]. Due to its strong dimerization constant, it has been employed for end functionalization of monomers to form telechelic polymers. Many telechelic polymers have been synthesized by end modification. Dimerization of ureidopyrimidinone takes place in two different states: DDAA and DADA (D-Donor, A-Acceptor). The state of dimerization depends upon the tautomeric form of ureidopyrimidinone. The tautomeric form of ureidopyrimidinone relies on the substitution at C-6 of isocytosine ring. Ureidopyrimidinone exist in three different tautomeric forms and are named with respect to its form: A) 6[1H]-pyrimidinone B) 4[1H]-pyrimidinone C) pyrimidin-4-ol (shown in Figure 2.14). Meijer et al. have reported that electron withdrawing group at C-6 position favours pyrimidin-4-ol tautomeric form in deuterated chloroform ($CDCl_3$) and 6[1H]-pyrimidinone in deuterated dimethyl sulfoxide (DMSO-d6) and alkyl/ether electron donating group favors 4[1H]-pyrimidinone in $CDCl_3$ and 6[1H]-pyrimidinone form in DMSO-d6.

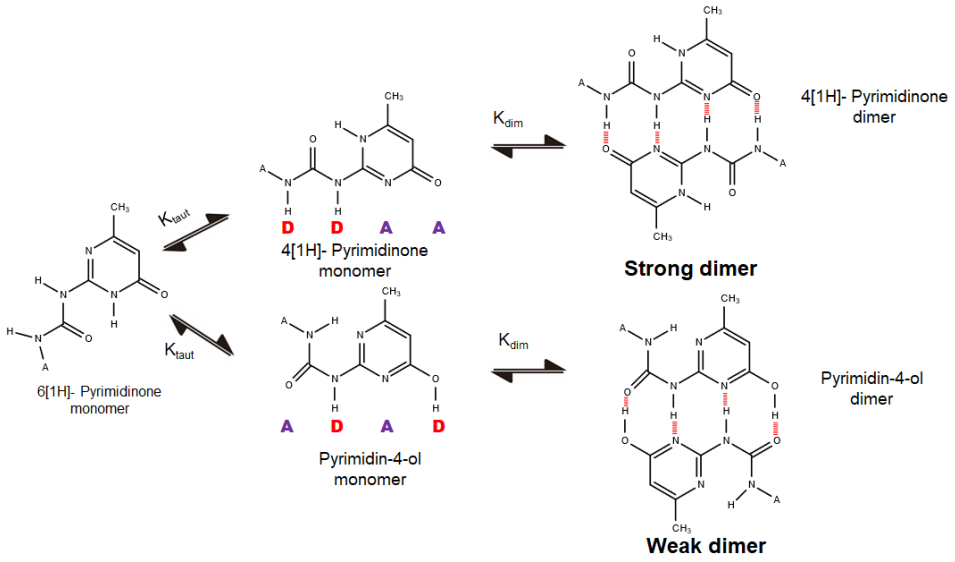

Figure 2.14 Tautomerization in Upy and its dimeric forms

The tautomeric form is also governed by solvent, temperature and radiation exposure. Ureidopyrimidinone exists in different tautomeric forms depending upon the type of solvent. In dimethyl sulfoxide (DMSO), molecules of Ureidopyrimidinone has been reported to exist in 6[1H]-pyrimidinone tautomeric form because of competition for hydrogen bonding with Upy and in 4[1H]-pyrimidinone and pyrimidin-4-ol tautomeric state when mixed in chloroform [38]. Apart from solvent, temperature also influence the tautomeric forms of ureidopyrimidinone. It has been reported that equilibrium of tautomeric form shifts with temperature and complete conversion from 4[1H]-pyrimidinone (keto) to pyrimidin-4-ol (enol) took place around 380 K and reversed back to 4[1H]-pyrimidinone form upon recrystallization in chloroform as shown in Figure 2.15 [39].

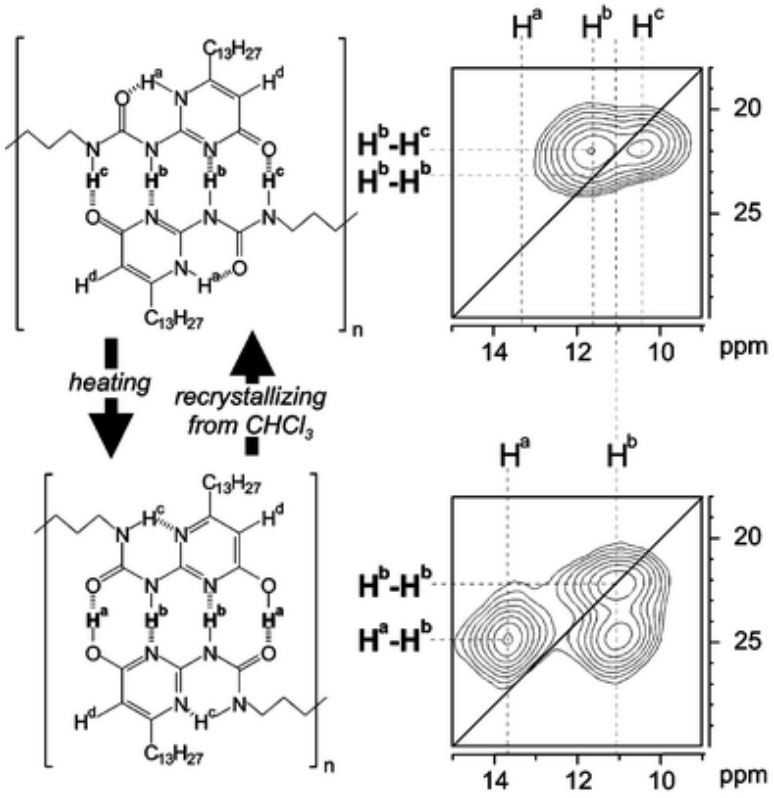

Figure 2.15 Temperature dependent tautomerization in ureidopyrimidinone and 1H double quantum mass spectrum [39]

Keto form of ureidopyrimidinone show stronger dimerization in comparison to enol form [39]. 4[1H]-pyrimidinone undergoes DDAA dimerization, whereas pyrimidin-4-ol follows DADA dimerization. In addition, Ureidopyrimidinone also undergoes tautomerism shift upon UV exposure. UV induced enolization has been reported in case of ureidopyrimidinone modified polymer [40].
Ureidopyrimidinone has been used to synthesize telechelic polymers as well as to introduce supramolecular interaction through Upy dimerization. Upy has been introduced in various polymeric systems via different methods as discussed in the following sections.

2.4.2 Strategies to substitute Upy in hydrophilic polymers

2.4.2.1 End functionalization

Upy has been substituted in monomers/oligomers to form telechelic hydrophilic polymer. Substitution of Upy on both ends of oligomers would allow the formation of cross-linked network in aqueous environment. End functionalized hydrophilic polymers have been studied to form nanoscale micellar structure and hydrogels in aqueous environment Meijer and co-workers have substituted Upy in PEG oligomers to form a cross-linked network for drug delivery application. Due to Upy dimerization, micellar structures with helical shaped fibres have been reported in aqueous solution [41]. It is however, difficult to form a hydrogel via end functionalization of Upy in hydrophilic polymers. In another study by Meijer and co-workers, a soft gel has been fabricated by acid base chemistry in a mixture of bi-functionalized and mono-functionalized PEG. Mono-functionalized PEG was unable to form hydrogel and exist in solution state due to weak transient networks. However, addition of bi-functionalized PEG had resulted in formation of a stable gel via pH shifting as shown in Figure 2.16. In the given studies, mixture was first mixed in basic medium to get a clear solution followed by addition of acid to form a phase separated viscoelastic hydrogel. Acidification cause conversion of enol to keto form, which form strong Upy dimerization and phase separation in the solution [42].

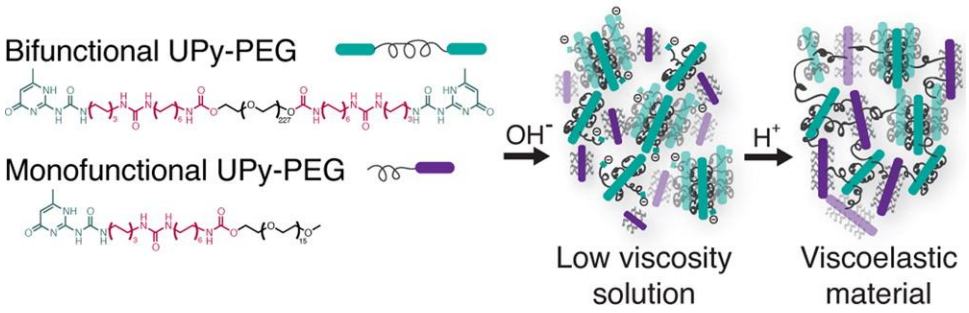

Figure 2.16 Hydrogel fabrication by mono and biofunctionalized Upy-PEG by pH switching [43]

Dankers et al. has reported the fabrication of a soft viscoelastic hydrogel from end-functionalized polyethylene glycol with storage modulus 100 Pa, but eventually dissolved when exposed to aqueous environment [44]. Conclusively, end functionalized oligomers required external cross-linker to form a hydrogel and unable to form hydrogel due to formation of weak transient networks in aqueous solution.

2.4.2.2 Pendant functionalization

Pendant functionalization is the most common functionalization exploited to form Upy substituted polymers by many researchers. Both synthetic and natural polymers have been functionalized in pendant fashion. The final properties of Upy substituted polymers varies with the properties of host polymer chain, concentration, Upy substitution, micro-environment and film fabrication method. Upy substitution in hydrophilic polymers have been reported to form robust hydrogels and shape memory properties. Upy functionalized poly (vinyl alcohol) has been synthesized by Zhou and co-workers to form a stretchable shape memory polymer. The strain at break and tensile strength of Upy functionalized PVA has increased from 10.5 MPa and 110%, for pure PVA to 12 MPa and 470%. However, the water adsorption decreases with increase in

Upy substitution due to hydrophobic nature of Upy as shown in Figure 2.17 [45].

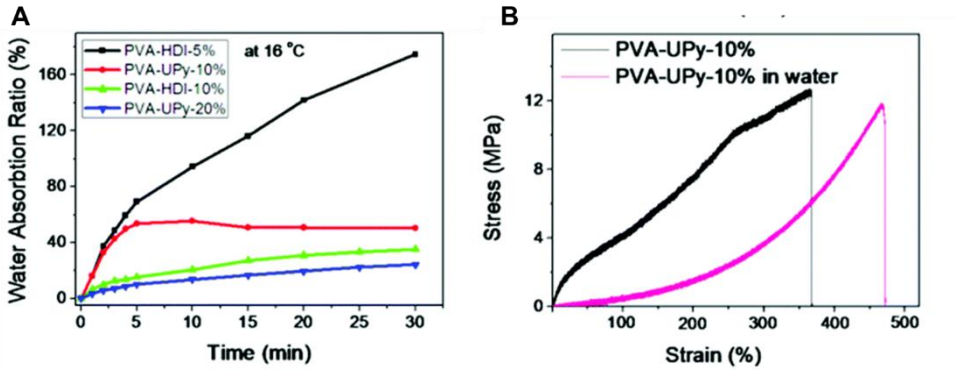

Figure 2.17 A) Water adsorption by hexamethylene diisocyanate cross-linked PVA (PVA-HDI) and PVA-Upy with different weight percent Upy substitution and B) stress strain curve of PVA-Upy in dry and hydrogel state [45]

In another study, substitution of Upy in dextran has resulted in a robust hydrogel with shear thinning and self-healing properties [46]. Substitution of Upy in hydrophobic polymers has been observed to enhance the viscoelastic properties of hydrogels and similar behavior has been observed in hydrophilic polymers as well. Poly (hydroxyethyl methacrylate) was substituted with Upy and has increased the viscoelastic properties. Increase in Upy substitution has increased the storage moduli from liquid to solid hydrogel and increase the zero-shear viscosity four times upon Upy substitution [47]. Conclusively, Upy substitution in polymer as pendant has transformed the properties of host polymer.

2.4.2.3 Copolymerization/Chain extension

Upy has been substituted in polymer chains during polymerization as a monomer/chain extension molecule. Substitution of Upy in polymer as block copolymer has introduced the elastomeric properties in comparison to end functionalized and pendant functionalized polymers. Chain extended (co)

polymer with polyethylene glycol as hydrophilic segment and Upy as hydrophobic self-dimerization segment has been reported to form a polymer with strong and resilience upon deformation properties, with 234 ± 6 MPa, yield strength in dry film state. Necking starts at 10% with >1100%, strain at break demonstrates the ductile properties as shown in section B of Figure 2.17. PEG-Upy chain extended (co) polymer based hydrogel has been fabricated by rehydration of solvent casted film. Cyclic stress strain tests of PEG-Upy chain extended (co) polymeric hydrogel till 500% strain have shown that a large hysteresis loss took place during first loading and almost no loss took place in the subsequent cycles as shown in section C of Figure 2.18 [48].

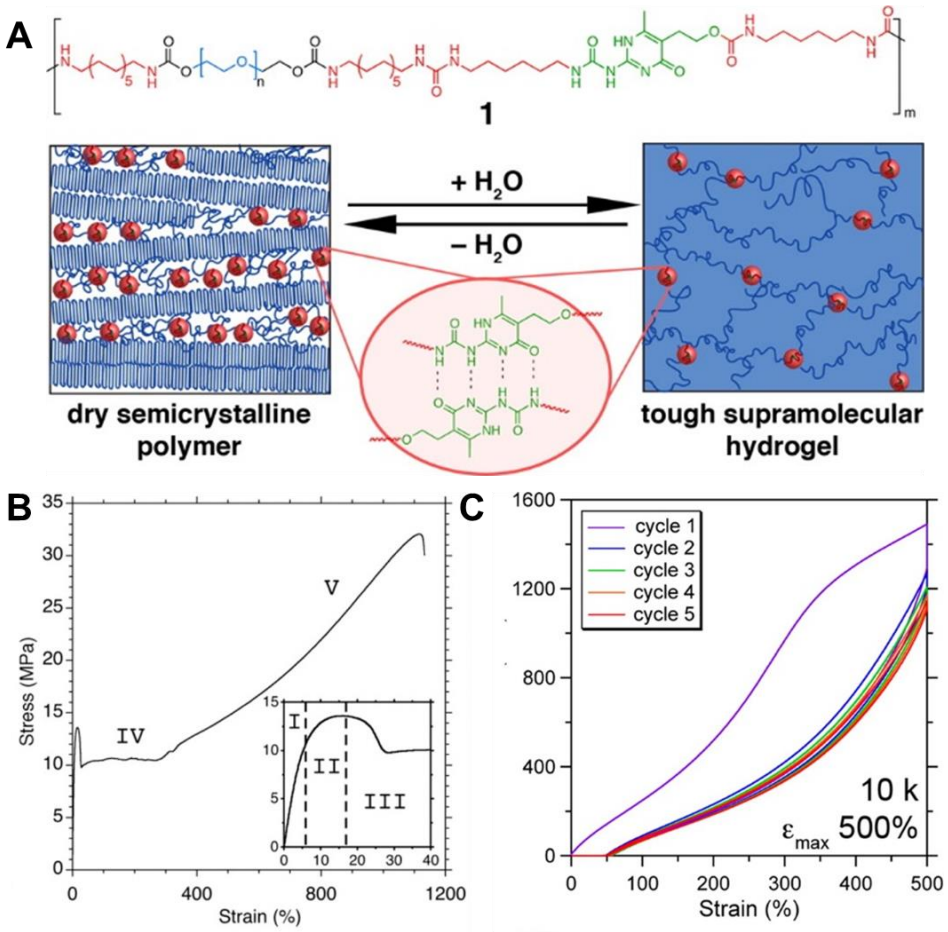

Figure 2.18 A) Schematics of PEG-Upy chain extended (co) polymeric hydrogel B) Stress strain curve for dry film of PEG-Upy chain extended (co)

polymer C) Cyclic stress strain curve for PEG-Upy chain extended (co) polymeric hydrogel [48]

Similarly, Upy substitution in the main chain in copolymer of polyethylene glycol: polycaprolactone (9:1) and polymer polyethylene glycol has been fabricated. Both polymers have been fabricated into a soft gel and had ~1000 Pa storage modulus [48]. In another study a block copolymer of polyethylene glycol, poly (caprolactone) and Upy has been synthesized to fabricate a viscoelastic hydrogel for intrarenal drug delivery application [44].

Among all the different Upy substitution strategies, pendant functionalization is the most preferred method over block copolymer and end functionalization due to direct substitution of ureidopyrimidinone on polymer backbone or sidechains.

2.4.3 Challenges with pendant functionalized polymers in hydrogel formation

2.4.3.1 Solubility

Hydrophilic polymers can be classified into two types depending upon solubility in water: a) water soluble hydrophilic polymers (WSHP) b) water insoluble hydrophilic polymers (WIHP). Hydrogel fabrication in WISP has been achieved either by solvent casting or by hot melt processing depending upon the host polymer, followed by hydration of the film. In solvent casting of Upy substituted WIHP, Upy substituted was dissolved in organic solvents which followed by solvent evaporation. Upy dimerization is solvent dependent and if the solvent favors the Upy dimerization, the resultant film would have efficient Upy crosslinking. However, substitution of Upy in WSHP has been reported to introduce non-directional hydrophobic associations which cause phase separation in aqueous solution due to hydrophobic nature of Upy.

Hydrophobic association among Upy moieties in aqueous environment prevents Upy dimerization. In most of the cases, hydrophobic interactions caused by Upy molecules in hydrophilic polymers has resulted in formation of micellar like structure or random aggregates. Complete dissolution of polymers in the solvent is required to allow the Upy dimerization and also the solvent should favor the Upy dimerization. Insolubility is an issue with the polymers which lack ionic interactions or functional group (which can form hydrogen bond with water) to counterbalance the hydrophobic effect of ureidopyrimidinone. It was observed that polymer like dextran, when substituted with Upy (10% substitution) has been suspended in phosphate buffer saline for hydrogel fabrication without phase separation. Dextran have hydroxyl groups, which can counter-balance the hydrophobic interactions [46]. However, in case of Upy substituted gelatin, it would undergo phase separation when suspended in aqueous solution and insolubility has been reported which prevents the hydrogel fabrication [49].

2.4.3.2 Lack of network formation

Hydrogel fabrication requires inter-chain crosslinking of polymers to form a network to hold water. Upy as a hydrophobic moiety can undergo self-dimerization that may result in formation of colloidal particles or intra-chain crosslinking. Intra-chain crosslinking and hydrophobic aggregation prevents the formation of network required for hydrogel fabrication.

2.4.3.3 Dual intermolecular interactions

In case of Upy modified water-soluble polymers, dual interactions were present including hydrogen bonding (Upy dimerization) and hydrophobic interactions (Hydrophobic Upy moiety). When suspended in aqueous solution, hydrophobic interaction and Upy dimerization compete. Upy dimerization relies on the events when two Upy moieties encounters, whereas hydrophobic aggregation

is spontaneous and took place instantaneously. Due to this, the resultant polymer lacks the supramolecular crosslinking requires to hold the structure and requires the addition of external cross-linker to support the hydrogel structure.

2.5 Hydrogel fabrication by supramolecular polymers

Researchers have been using the conventional methods for hydrogel fabrication of supramolecular polymers due to limited development in the area of methods development. Supramolecular cross-linked network formation requires a dynamic and a favorable microenvironment to form hydrogel. Method of crosslinking should vary with the type of supramolecular interaction involved in crosslinking.

2.5.1 Direct hydrogel fabrication via liquid phase

Supramolecular hydrogels have been fabricated in liquid phase and are able to form cross-linked network in the aqueous phase. This method requires the dissolution of polymer in liquid phase followed by gelation. Gelation requires the formation of network formed by non-covalent crosslinking of functional groups. Homogeneous suspension of polymer is a prerequisite for this method. Non-covalent interactions like hydrogen bonding, metal-ligand complexes, hydrophobic interactions, inclusion complexes and ionic crosslinking have been utilized for the fabrication of hydrogels. Aure´lia et al. has studied the cyclodextrin and adamantane modified hyaluronic acid (HA) to form inclusion complexes in aqueous environment. Modified HA has formed micro-gels at low concentration and a viscoelastic gel above threshold concentration (1.7 g/L) [50]. Jang et al. has reported the ionic crosslinking in alginate polymer solution via Ca^{2+} ion crosslinking. Incubation for 10-12 hours at $37^{o}C$ has formed a stable hydrogel [51]. In case of Upy modified polymers, pH switching has been used for the hydrogel fabrication in liquid phase. Meijer et al. has reported the

fabrication of end and bi-functionalized PEG-Upy via pH switching via acidification of base polymer solution [42].

2.5.2 Hydrogel fabrication via solid phase

Hydrophilic polymers which are insoluble in aqueous solution are not able to form hydrogel in aqueous phase. For such polymers, the first most basic strategy to form a hydrogel is solvent casting. Polymer was dissolved in organic/inorganic solvent followed by solvent evaporation to form a dry film. Further the dry film as rehydrated to form the hydrogel. In case of supramolecular polymeric systems, the supramolecular interactions formation majorly relies on the solvent microenvironment. If the solvent system used for film fabrication permit supramolecular interaction and self-assembly takes place during evaporation of the solvent, it would result in the formation of supramolecular crosslinks in the polymeric film. However, in majority of the systems the supramolecular polymers were able to form limited supramolecular crosslinks which would result in a weakly cross-linked film. Zhou et. al. has studied the Upy substituted poly (vinyl alcohol) polymer system and has fabricated the film by solvent casting in N-methyl-2-pyrrolidone (NMP) to get a film, which was rehydrated to form a stretchable hydrogel with 13 MPa, tensile strength and 370%, strain at break [45]. Another common process for the water insoluble polymers to form a hydrogel is by hot press moulding. In this process, the polymer was hot pressed into the mould shape. Christopher et al. has fabricated the hydrogel of Upy substituted poly(hydroxyethyl methacrylate) through hot press moulding at 135^{o}C [47].

2.6 Improved strategies for supramolecular hydrogel fabrication

In supramolecular polymeric hydrogels, non-covalent crosslinks formation plays a vital role in strengthening the structure of hydrogels. Hydrogels would not be able to gain the required strength if they are not able to form the threshold

crosslinking required to stabilize the hydrogels system. Several new methods have been developed by the researchers to form a stable hydrogel. Feng et al. has reported a new method for fabrication of a stretchable hydrogel through solvent exchange via dialysis, shown in Figure 2.19. In the given studies, Phenyl isothiocyanate and 1-adamantyl isothiocyanate was used to modify the gelatin biopolymer in DMSO (Dimethyl sulphoxide). After 5 hours of reaction, the reaction mixture was dialyzed with distilled water. Hydrophobic crosslinking took place in a controlled way in a dynamic micro-environment to form a stretchable hydrogel. Unreacted functional groups have been reported to play an important role in formation of a stable and stretchable hydrogel. In absence of unreacted phenyl and adamantly group, the gel does not have sufficient crosslinking to maintain its integrity [33].

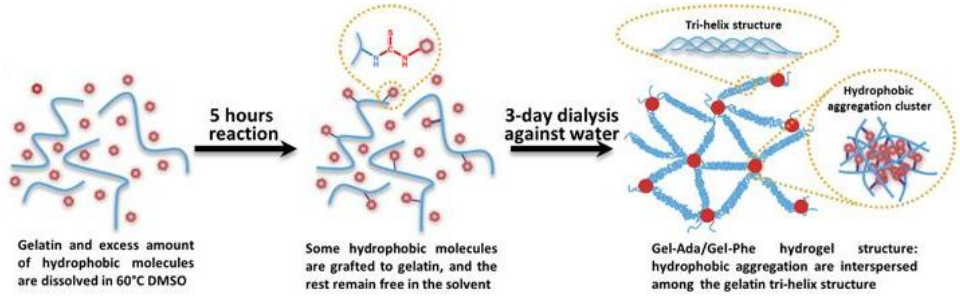

Figure 2.19 Hydrogel fabrication of Phenyl and adamantyl modified gelatin via dialysis of the reaction mixture through dialysis of the reaction mixture [33].

Another method has been used by Meijer and co-workers to fabricate a hydrogel in Upy modified PEG-PCL-Upy shown in Figure 2.20. Upy modified PEG-PCL-Upy was suspended in 50% THF to form a ternary mixture, followed by evaporation of THF by continuous stirring with heating. In this study, water-THF system provides a dynamic micro-environment required to form supramolecular crosslinking between Upy molecules followed by removal of THF to form a hydrogel [44].

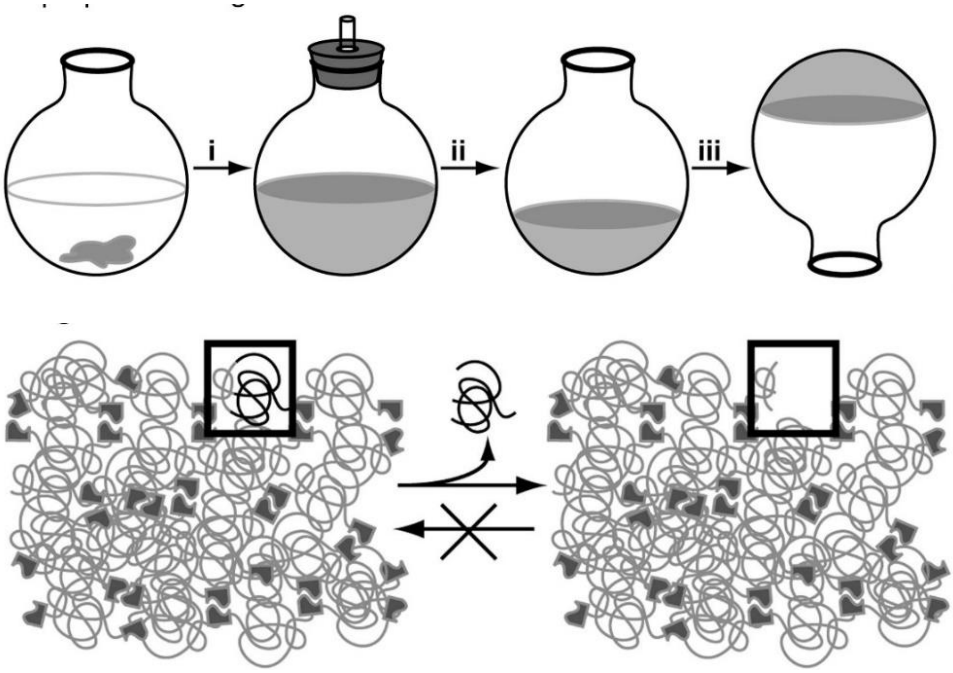

Figure 2.20 Hydrogel fabrication method for the PFG-PCL-Upy polymer via dissolution in 50% THF (i) followed by removal of THF (ii) to form a viscoelastic gel (iii). [44]

Conclusively, literature review demonstrates the gelatin based hydrogels as a potential biomaterial for biomedical application. Since, the applicability of gelatin hydrogel was limited by its low mechanical properties due to limited crosslinking, it was either cross-linked chemically and enzymatically or mixed with other synthetic polymers to form double network hydrogels. Supramolecular crosslinking has been introduced in polymeric hydrogels via Upy substitution to strengthen the mechanical properties. Upy has not been studied in hydrophilic polymeric systems and requires an extensive study to explore its potential in hydrophilic polymers. In addition, the fabrication strategies available for the fabrication of supramolecular hydrogels is limited and are not able to facilitate the formation of supramolecular crosslinks.

2.7 Summary

From the literature, gelatin has been regarded as a potential biopolymer and forms a weak and brittle hydrogel below sol-gel temperature due to hydrogen bonding. As gelatin is derived from the hydrolysis of collagen, it lost most of its covalent and physical crosslinks required to maintain the integrity of the structure. For it to be useful with sufficient mechanical integrity, it has been cross-linked chemically and enzymatically to introduce covalent crosslinking. However, apart from covalent crosslinking, non-covalent interaction plays a major role in natural structures and systems. Non-covalent/ supramolecular crosslinking has been introduced in the polymeric system via substituting self-assembled moieties in polymeric systems. Upy is a self-assembled moiety, which can undergo self-dimerization via hydrogen bonding. It has been substituted in many synthetic and natural polymers to introduce supramolecular cross-linking. Upy substitution in protein based polymers like gelatin has not been explored to fabricate a stretchable hydrogel. Therefore, it is in the interest of this thesis to explore Upy substitution in gelatin biopolymers as well as device a new fabrication method for hydrogel fabrication. The scope will cover the effect of water-THF co-solvent system on structural and chemical interactions in gelatin and Upy substituted gelatin biopolymer. Through these studies, a gelatin based elastomeric hydrogel will be fabricated with potential biomedical application and would replace the synthetic hydrogels.

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Chapter 3

Experimental Methodology

This chapter demonstrates the experimental procedures and characterization techniques used for the project. Sections are divided among the co-solvent optimization techniques, film fabrication and elucidation of film fabrication by various techniques including electron microscopy, Nuclear magnetic resonance spectroscopy, mechanical testing and swelling studies.

3.1 Materials and reagents

Gelatin A (300 bloom), 2-amino-4-hydroxyl-6 methyl pyrimidine, 1, 6-diisocyanatohexane, pyridine, hexane, tetrahydrofuran, ethanol, deuterated chloroform, deuterium oxide and deuterated dimethyl sulfoxide were procured from Sigma-Aldrich.

3.2 Solubility analysis of gelatin and Upy substituted gelatin derivatives in water and water-THF/ethanol co-solvent system

Transmittance analysis was carried out to test the solubility analysis of the gelatin/ ureidopyrimidinone substituted gelatin derivatives in distilled water or cosolvent system (water-THF/ethanol) using Agilent UV-Vis NIR Spectrophotometer (Cary 5000) 0.10, 1.00 and 10.00 mg of gelatin/ ureidopyrimidinone substituted gelatin derivatives were mixed in the desired 1 ml of solvent (distilled water/ cosolvent) and was continuously stirred to get a homogeneous solution or suspension. The solution was then loaded in 36 well plates for transmittance at 600 nm using plate reader.

For *in situ* analysis, 20 mg of gelatin and Upy substituted gelatin derivatives were mixed in 1 ml distilled water to make 20 mg/ml concentration for 6 hours at 60^{o}C with continuous stirring to get a homogeneous initial aqueous solution/suspension. Further, the samples were diluted with water/THF/ethanol by adding 100 µl of water/THF/ethanol 10 times stepwise till concentration reaches 10 mg/ml and was mixed at each step for 5 min using pipette inside cuvette and transmittance at 600 nm was measured for transmittance analysis. Transmittance was measured at 600 nm for turbidity measurement of samples. Transmittance follows the law of conservation of energy for non-fluorescent materials and values lies in the range of 0-1. Transmittance, $\tau(\lambda)$ is defined by the ratio of radiant flux, ϕ_t (λ) transmitted by the sources to the incident radiant flux, ϕ_i (λ) in equation 3.1:

$$\tau(\lambda) = \frac{\phi_t(\lambda)}{\phi_i(\lambda)} \quad \textbf{(3.1)}$$

3.3 Structural analysis of gelatin and Upy substituted gelatin derivatives for structural transformation

Scanning electron microscopy was carried out to study the micro-structural analysis of gelatin when mixed in water and water-THF cosolvent system. For microstructure analysis, gelatin and Upy substituted gelatin derivative (GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50) were mixed in 1 ml distilled water/ x % THF at 70^{o}C with continuous stirring using magnetic stirrer hot plate to get a homogeneous suspension/solution with 10 mg/ml concentration. 200 µl of samples was transferred to vials and were kept in deep fridge at -80^{o}C for 10-12 hours and was transferred to freeze dryer to get freeze dried samples. Further freeze-dried samples were loaded on the sample holder using two-sided carbon tape and was loaded in the sample holder of FESEM 7600F. Samples were observed at 5 kV at different magnifications.

3.4 Chemical analysis of gelatin Upy substituted gelatin derivatives

Chemical analysis of gelatin and ureidopyrimidinone substituted gelatin derivatives was carried out to do qualitative measurement for change in chemical structure. Fourier transform infrared spectroscopy (ATR-FTIR) and ^{1}H Nuclear magnetic resonance spectroscopy (NMR) was carried out for chemical analysis of samples.

3.4.1 Fourier transform infrared spectroscopy (ATR-FTIR)

Ureidopyrimidinone, Gelatin and ureidopyrimidinone substituted gelatin derivatives were characterized by ATR-FTIR spectroscopy for their chemical

structure and purity of the samples using PerkinElmer fourier transform infrared (FTIR) spectrometer (Frontier). For ureidopyrimidinone, pellet was made using potassium bromide (KBr), whereas freeze dried powders/film were mounted directly over the Zinc selenide (ZnSe) crystal and were scanned multiple times (50 scans) by infrared rays with wavenumber range 400-4000 cm^{-1} by the instrument to obtain the emission/absorption spectrum. During scanning, samples were exposed to monochromatic infrared waves of different wavelength, absorbed by the infra-red active organic functional group of samples. Dipole moment of such infrared active functional groups changes as it undergoes expansion and contraction. Absorption of IR radiation by infrared active groups would result in different modes of vibrations depending upon the stiffness of the bonds and the masses of atoms at each end of bond. Also, a molecule would only absorb radiation if the incoming radiation is of the similar frequency as of the fundamental modes of vibration of the molecule. Following are the different modes of vibration of molecules – Asymmetric and symmetric stretching, out-of-plane and in-plane bending, deformation, rocking, wagging and twisting. FTIR-analysis can be interfered by the moisture present in the samples. Therefore, proper drying of all the samples is necessary to eliminate moisture effect.

The relationship between IR intensity and dipole moment is following equation 3.2:

$$I_{IR}\ \alpha\ \left(\frac{d\mu}{dQ}\right)^2 \qquad \textbf{(3.2)}$$

‘μ’ is the dipole moment and ‘Q’ is the vibrational coordinate.

3.4.2 ^{1}H Nuclear magnetic resonance spectroscopy (NMR)

Ureidopyrimidinone, gelatin and ureidopyrimidinone substituted gelatin derivatives were characterized by NMR spectroscopy for chemical analysis using AVANCE I 400 MHz spectrometer. For ^{1}H NMR spectral analysis of ureidopyrimidinone, sample was prepared by mixing 10mg of ureidopyrimidinone powder in 700 µl deuterated chloroform (Sigma-Aldrich). Gelatin and ureidopyrimidinone substituted gelatin derivatives samples were prepared by mixing 10 mg of powder sample in deuterated dimethyl sulfoxide (Cambridge Isotope laboratories, Inc). Further samples were loaded in NMR tubes to obtain ^{1}H NMR spectrum. Samples were scanned from 0 ppm to 10 ppm at 400 MHz frequency.

NMR spectroscopy works on the principle that most of the nuclei have spin and are electrically charged. Under the influence of an external magnetic field, transfer of energy is feasible between higher energy level and base energy level (shown in Figure 3.1). When the spin returns to base level, energy is emitted at a certain wavelength of radio frequencies due to transfer of energy. The signal that has a match with this transfer is measured to generate NMR spectrum for the concerned nucleus.

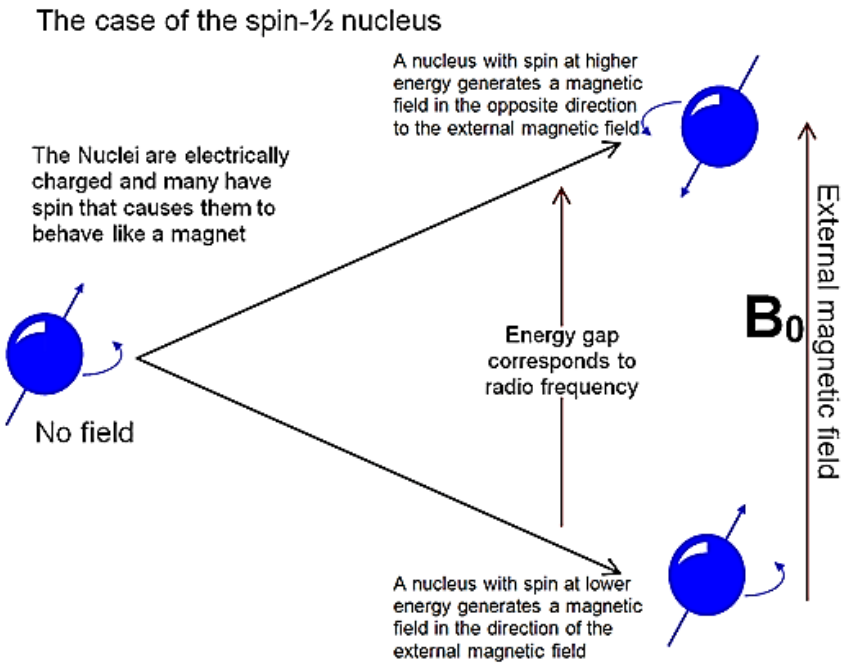

Figure 3.1. Principle of NMR spectroscopy demonstrating the effect of external magnetic field on the spins of nucleus and energy gap

3.5 Elastomeric film fabrication and characterization

3.5.1 Film fabrication

One gram of biopolymer (gelatin/ureidopyrimidinone substituted gelatin) was mixed in 20 ml of distilled water and was continuously stirred at 60-70^{o}C to form a homogeneous suspension. After obtaining a homogeneous suspension, 20 ml of tetrahydrofuran was added to the sample dropwise and was continuously stirred at room temperature until the colour of the suspension changed from milky white to clear. Further 60 ml of tetrahydrofuran was added slowly to the solution to make the final co-solvent volume to 100 ml and concentration of 10 mg/ml. Addition of THF would change the colour of the solution from clear to milky white again. The obtained milky suspension was incubated at 37^{o}C for 24 hours. After 24 hours, the biopolymer was sedimented at the bottom to form elastomeric film and a clear supernatant at the top of the

film. Supernatant was removed and the film was peeled off carefully from the container for further processing and studies.

3.5.2 Physical and structural analysis of elastomeric films

3.5.2.1Optical microscopy

Elastomeric film obtained from the film fabrication at 80% THF was investigated for its structure using optical microscope at 10X resolution using Nikon 80i eclipse upright microscope. Film was mounted on the glass slide and was directly observed for its structure. For cross-section view, film cross-section was cut using surgery blade and was mounted on glass slide for observation using the microscope.

3.5.2.2 Scanning electron microscopy of elastomeric films

Gelatin and GEUPYII (0.50) elastomeric films obtained at 80% THF were observed in scanning electron microscope for its microstructural analysis. Films obtained were cut into pieces with dimension ~2mm x 3mm and were kept at -80^{o}C for freezing. Samples were further transferred to freeze dryer to get the dry film. For cross-section analysis, dried films were broken manually and were mounted on half stub for cross-section structural analysis. As the samples were non-conductive, they were sputter coated with gold atoms in a sputter coater for 45 seconds at 40 milli ampere. Further samples were mounted over the sample holder using carbon tape to load the sample inside JEOL FESEM 7600F scanning electron microscope. Samples were analysed at 5 kV at 6.00 mm working distance at low and high magnification to get SEM images of the films. For quantification of pore size analysis in films, the SEM images were processed using IMAGEJ 1.52o software and the pore diameters were calculated and were plotted using.

Using scanning electron microscopy, high magnification images can be achieved due to lower wavelength of electrons in comparison to optical light microscopy which uses light to produce images.

Microscopic resolution or the resolving power of a microscopy is the shortest distance between the two points which can be distinguished as separate entities and is related to wavelength by the following equation 3.3:

$$Resolving\ power = \frac{\lambda, wavelength}{Numerical\ aperture\ of\ objective\ lens} \quad \textbf{(3.3)}$$

In SEM, two types of electron sources including thermionic source and field emission source are used to produce an electron beam as shown in Figure 3.2. In thermionic sources, the filament is made up of materials like tungsten, lanthanum hexaboride etc., which will produce electron when heated above threshold, whereas electron emission takes place due to large electric potential gradient in case of field emission gun as shown in Figure 3.2. The electron beam produced is condensed by the condenser electromagnetic lenses to remove the high angle electrons. Further deflecting coils and objective lens assist in focussing the beam over the desired region of samples. Interaction of electron beam with the samples surface would produce auger electron, backscattered electrons, X-rays and secondary electrons. Backscattered and secondary electrons are detected by the detector for image formation. Image from the secondary electrons can provide the topographic information, whereas the image from backscattered electrons provide topographic information in addition to crystallographic and magnetic field information.

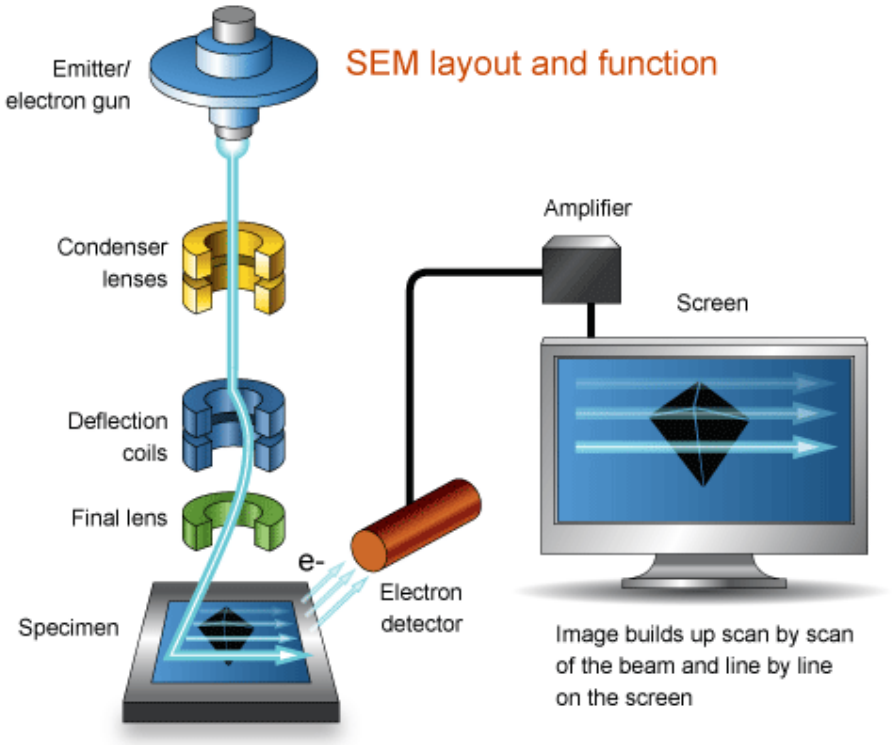

Figure 3.2. Scanning electron microscope layout

3.5.3 Solvent exchange from the elastomeric films

Water-THF co-solvent from the films fabricated at 80% THF was exchanged with distilled water by two methods. In the first method, the films were kept in distilled water for 72 hours and suspended water was replaced after every 24 hours. In the second method, films were dried at 38^{o}C under vacuum for 48 hours to remove the solvent from the films. Further, air dried films were rehydrated with distilled water to make hydrogel.

3.5.4 Solvent content analysis of elastomeric films

To analyse the solvent content of 80% THF film, solvent exchange films and rehydrated air-dried film of gelatin and GEUPYII (0.50) were analysed using TGA TA Instruments Q500 for thermal gravimetric analysis. For analysis, the films were cut into small pieces with weight >20 mg and loaded into the machine for analysis with nitrogen pressure of 50 ml/min flow rate into the sample chamber. Further, the weight change of the sample was observed with rise in temperature to 200^{o}C at a rate of 5^{o}C/min. Thermal gravimetric analysis measures the change in the weight change of the sample when heated at constant rate due to volatile components and decompositions.

3.5.5 Analysis of mechanical properties

Elastomeric films were characterized for their mechanical properties using MTS mechanical tester Criterion Model 42. Films were cut into standard dumbbell shaped films using cutter. The samples have a gauge length of 9.53 mm and width at gauge length of 3.18 mm. After sample preparation, thickness of the samples was measured using micro-meter screw gauge. Further, samples were loaded onto the tensile fixtures with 50 N load and tested at a cross-head speed of 1mm/sec. Precaution should be taken to ensure that the sample does not slip from the grippers and failure takes place only within the gauge length. Strain at break and the Young's modulus was calculated by testing triplicates of the samples. The Young's modulus was calculated by origin software by using slope of initial part of the curve by using the following equation 3.4:

$$Young's\ modulus\ (\mathrm{E}) = \frac{Stress}{Strain} = \frac{F/A}{\Delta l/l} \qquad \textbf{(3.4)}$$

F -Force required for failure of sample; A - cross-sectional area of test sample; Δl – sample extension length; l - sample original length.

3.5.6 Elucidation of assembly of elastomeric film fabrication

To elucidate the film fabrication mechanism, point before cloud point was taken, which was 43.8% THF for gelatin and 50% THF for GEUPYII (0.50). 100 µl of gelatin and GEUPYII (0.50) samples were prepared with 43.8% THF and 50% THF (concentration - 10 mg/ml), respectively. In case of gelatin, THF proportion was adjusted from 43.8% THF to 60% and 80% THF by addition of THF. In the case of GEUPYII (0.50), THF proportion was adjusted from 50% to 52.5%, 60% and 70% THF by addition of THF. After THF proportion adjustment, samples were mixed for 30 seconds with pipette manually and were transferred for deep freeze at -80^{o}C for 10-12 hours.

Freeze dried samples were characterized by ATR-FTIR and FESEM for their chemical and structural analysis by similar process as described earlier.

3.6 Statistical analysis

Triplicates of the samples were prepared, and the experimental data obtained from the experiments are expressed as mean± standard deviations. Origin software was used to plot the data and statistical analysis.

Chapter 4

Fabrication of stretchable gelatin gel via co-solvent system

This chapter describes the structural and chemical transformation by co-solvent systems on gelatin biopolymer, followed by fabrication of gelatin-based elastomer and its structural, physical and mechanical properties. The chapter will start with a brief introduction and material and methods, followed by the optimization of co-solvent system studies in section 4.3.1 (Results and discussions). The section 4.3.2 (Results and discussions) presents the co-solvent based structural transformations. After co-solvent optimization, section 4.3.3 (Results and discussions) represents the fabrication methods to achieve a stretchable elastomeric film. Section 4.3.4 introduces the chemical and structural changes took place during film fabrication.

4.1 Introduction

Gelation is a biopolymer derived from the hydrolysis of animal collagen. It has diverse application in food, pharma and biomedical industries due to its biodegradability and biocompatibility [1]. The chemical composition of gelatin is almost like collagen except the changes made by the hydrolysis methods. During hydrolysis of collagen, the structure changes from triple helix ordered structure to random coils as reported in literature review [2]. Gelatin undergoes sol-gel transformation below transition temperature i.e. 20-25^{o}C and forms a thermo-sensitive gel [3]. The random coils of gelatin biopolymer have the property to undergo renaturation upon cooling to form double/triple helixes. Formation of double/triple helixes involves multiple coils forming a 3-D network and can hold water molecules due to hydrophilic properties [4]. Gelatin does form a hydrophilic network, however it has hydrophobic segments as well. The tripeptide repeat Glycine-x-y forms a hydrophobic segment of gelatin when x and y are hydrophobic amino acids (usually x- proline and y – hydroxyproline). The hydrophobic segments are distributed throughout hydrophilic segments. Due to both hydrophobic and hydrophilic segments, gelatin has been considered as hydrocolloids [5]. Hydrophilic segments and charged amino acid are held responsible for the stability of gelatin in aqueous environment, however hydrophobic segments provide rigidity and stability to the helical structures of gelatin [6]. Due to hydrophobic segments, gelatin has been considered as hydrocolloid [7].

Coacervates are colloid rich liquids which separates from colloidal solution when exposed to dehydrating agents like temperature, pH, electrolytes and non-solvent. Macromolecules like proteins, synthetic polymers, biopolymers, drugs, polysaccharides have been known to undergo coacervation [8]. It has been exploited for microencapsulation of molecules in pharmaceutical industries, agriculture, food, printing, cosmetics, waste water treatment and protein purification [9-12]. Coacervation has been studied in biopolymers for forming

porous scaffolds and nanoparticles [13, 14]. In case of gelatin, nanoparticles have been fabricated by using ethanol as non-solvent. Groves et al has studied the effect of pH and ethanol proportion on the aqueous solution of gelatin. The coacervation/phase separation takes place upon addition of ethanol in aqueous solution with pH 5 and 7 from the beginning, whereas forms a stable clear solution at pH 3 till 70% THF. The nanoparticles with size 1082.1 nm and 500.6 nm have been reported to form at 50% and 45% ethanol in gelatin solution at pH 7 [13]. Similar gelatin nanoparticles have been reported upon exposure of aqueous gelatin solution to acetone as non-solvent [15]. In the abovementioned studies the particle formation takes place using non-solvent for coacervation followed by freeze drying of particles. The abovementioned coacervates of gelatin in water-ethanol co-solvent system was formed due to electrostatic and hydrophobic interactions of gelatin [16, 17]. The polarity of the non-solvent employed for coacervation plays an important role in coacervation process [18].

Coacervation has been considered as a system of weakly interconnected network system which has been associated due to high concentration [19]. However, though the coacervates in the above-mentioned studies have been studied to form nano/micro particles, they have been reported to form interconnected network structure as well. If the interactions between the coacervates is strong enough, they form an interconnected network structure. The formation of interconnected network system depends on polymer-polymer, solvent-solvent and polymer-solvent interactions [20, 21]. Arvidson et al. has reported the formation of thermo-sensitive gel of methylcellulose via phase separation [22]. Stretchable hydrogel fabrication has been reported by Haragachi and co-workers. Nano-composite gel of clay, N, N-dimethyl (acrylamidopropyl) ammonium propanesulfonate and butanesulfonate was fabricated via phase separation with 1000-2000%, strain at break [23]. If the deformation time of the phase separation is slower than relaxation time of the coacervates, the stress of the coacervates get eventually divided among them to form interconnected network and they will eventually connect with each other

along with coarsening [24]. In this work, we studied the use of tetrahydrofuran (aprotic) and ethanol (protic) as a non-solvent system to fabricate a gelatin based elastomeric gel and studied the structural transformation.

4.2 Experimental methods

4.2.1 Solubility of gelatin in co-solvent system

20 mg of gelatin was mixed in 10 ml distilled water at 60^{o}C for 6 hours to get a clear solution. To study the coacervation, 100 µL aliquots of co-solvent (THF/ethanol) was added to the aqueous solution of gelatin stepwise till cloud point. After every step, solution was mixed for 5 minutes to stabilize and the transmittance of the solution was measured at 600 nm using UV-VIS spectrophotometer.

After phase separation, the gelatin biopolymer coacervates sediments to form a film. To quantify the proportion of gelatin sedimented in the film state and supernatant, film and supernatant were separated and freeze dried to quantify the gelatin proportion in the film and supernatant.

4.2.2 Fabrication of gelatin gel

Gelatin was mixed in distilled water at 60^{o}C for 6 hours to get a clear solution. Co-solvent was added to the solution to make it 40% and mixed at room temperature to form a stable clear solution. Further, the co-solvent (THF/ethanol) was adjusted to 50% and 80% co-solvent proportion and was incubated overnight at 37^{o}C to form a film. After incubation, the solution turned from milky to clear solution and the film was peeled off from the bottom of the container for mechanical and chemical analysis.

4.2.3 Structural and chemical analysis of gelatin in THF

Gelatin was mixed in distilled water at 60^{o}C for 6 hours to get a clear solution. THF was added in with respect to the initial volume to make 43.8% THF solution and was mixed together to form a clear solution. Further the THF proportion of the samples was adjusted to 60% and 80% THF. The samples were mixed for 30 seconds using pipette and was freeze dried to get the powder sample.

4.3 Principal outcomes

4.3.1 Solubility analysis of gelatin in water and co-solvent system

Gelatin is a thermo-sensitive polymer which forms a gel below sol-gel transition temperature. It formed a homogeneous aqueous solution with >90% transmittance in water above transition temperature and has been reported to be insoluble in organic solvents. Hydrophilic segments and charged functional groups present in gelatin are responsible for good solubility in water [25]. Exposing the gelatin solution to different co-solvents usually causes increase in turbidity due to coacervation. Gelatin has been reported to form coacervates depending upon the pH of the solution. Groves at al. has reported an increase in turbidity with increase in ethanol concentration at pH 5/7 and no increase in turbidity at pH 3 till 70% ethanol [13]. The stability of gelatin in co-solvent system varies with the electronic state of gelatin biopolymer. In this work, solubility of gelatin in ethanol and tetrahydrofuran as co-solvents has been studied. In dilution studies, it was found that the aqueous solution of gelatin would undergo phase separation upon dilution with ethanol and tetrahydrofuran. In our study, gelatin underwent a phase separation at 50% ethanol and 47% tetrahydrofuran, demonstrated by transmittance analysis in Figure 4.1. Phase separation/coacervation took place early for the tetrahydrofuran in comparison to ethanol probably due to lower polarity of THF in comparison to ethanol. The

relative polarity of THF and ethanol is 0.207 and 0.654, respectively [26]. Also, THF is an aprotic solvent and ethanol is a protic solvent, which could be responsible for the difference in phase separation.

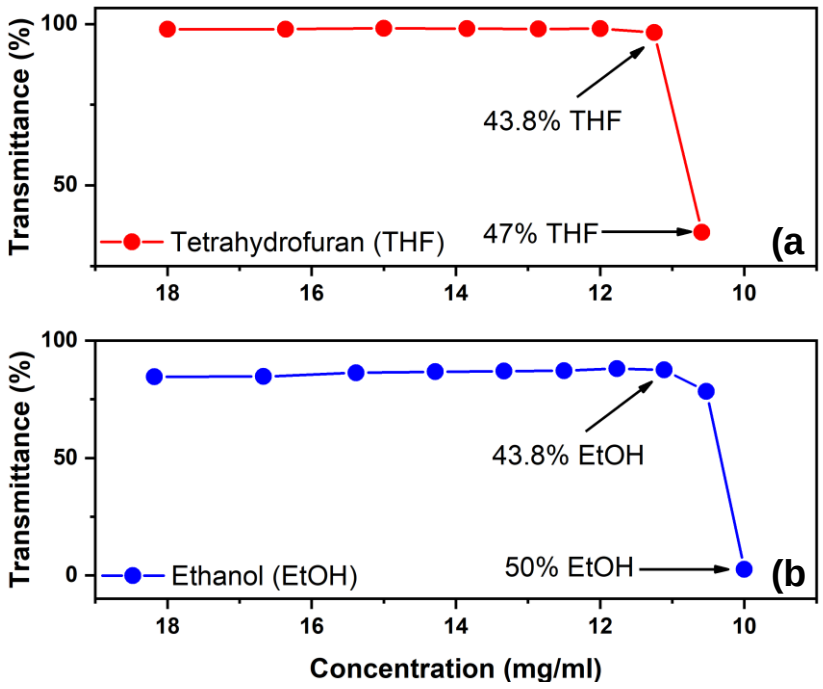

Figure 4.1. Transmittance analysis of gelatin aqueous solution *in situ* with different proportion of a) THF b) ethanol

4.3.2 Structural analysis of gelatin in water and co-solvent system

From *in situ* transmittance analysis of gelatin, it was observed that the cloud points for aqueous solution of gelatin were at 47% THF and 50% ethanol, respectively. Hence, 43.8% and 50% co-solvent (THF/ethanol) proportion were chosen to do the structural analysis of stable state and phase separation states.. In case of 43.8% co-solvent (THF/ethanol) proportion, gelatin solutions were stable and underwent phase separation when the THF was shifted to 50% THF. At 43.8% co-solvent proportion, it was observed that both co-solvent solutions

in THF and ethanol forms a clear solution and have almost similar structural characteristics when freeze dried powders were observed through scanning electron microscopy shown in Figure 4.2. In 43.8% ethanol and THF solutions, the gelatin was present as spherical particles. The spherical structures have formed due to the presence of moving droplet phase (MDP), which forms during the phase separation in polymeric solutions [24]. This MDP could undergo coalescence and forms larger coacervates. Similar particle formation has been reported in ethanol water and THF water solutions of polymers [16, 27]. However, solutions with 50% co-solvent proportion have undergone phase separation as shown in transmittance studies but turned clear after incubation. In the solution phase of 50% ethanol, spherical particles were present and formed a fused structure. Xiaohua et al. has also reported the formation of fused structures in gelatin at 50% ethanol [27].

To study the coacervation in gelatin by tetrahydrofuran, gelatin aqueous solution was adjusted to 50% THF by addition of tetrahydrofuran and was incubated overnight at 37^{o}C. The solution colour changed from milky to transparent due to sedimentation of gelatin coacervates after overnight incubation. The sedimented translucent film and the supernatant solution were separated in two glass bottles, followed by freeze drying. The freeze dried sedimented film and the supernatant was observed by scanning electron microscopy for their structural transformations. In supernatant at 50% THF, poly-dispersed fused coacervates were present, whereas the film had micro-porous structure with 150-350 nm pore size as shown in Figure 4.3. Spherical shape of the coacervates is due to the presence of inhomogeneous micro-domains in water-THF co-solvent system. Zu et al. has reported that blending of THF with aqueous solution forms a nanoscale droplet [28]. The fused structure of gelatin coacervates could be due to coalescence of coacervates. Increase in THF proportion could have decreased the gelatin-water interactions and enhanced the water-THF interaction which may have caused the formation of film. Similar effect had been observed by Han and coworkers [29]. Apart

from this, the sedimented coacervates at 50% THF forms a micro-porous film. Similar porous structured films have been formed when the polymeric film has been exposed to non-solvent [30].

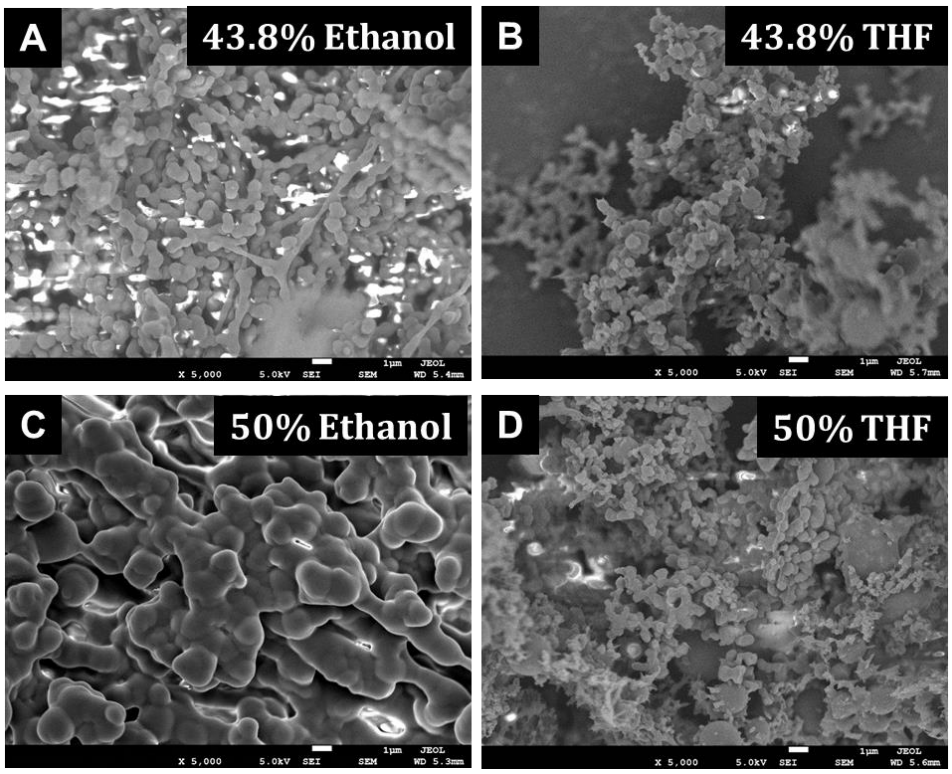

Figure 4.2. SEM micrographs of freeze-dried gelatin in A) 43.8% ethanol B) 43.8% THF C) 50% ethanol d) 50% THF (Gelatin concentration – 10 mg/ml)

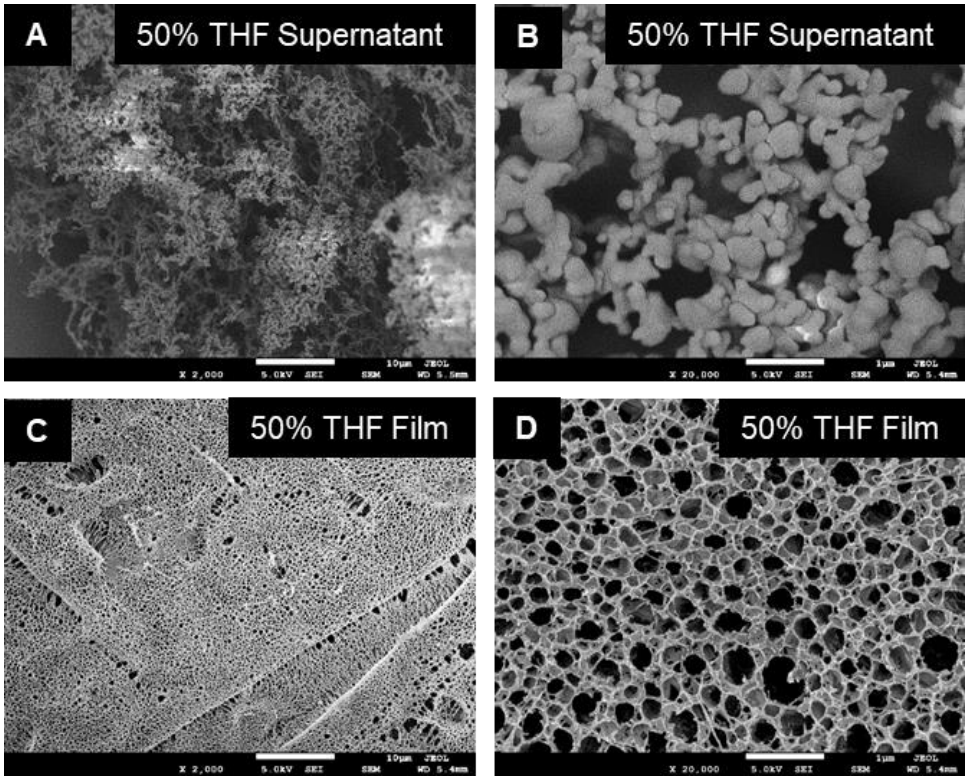

Figure 4.3. SEM micrographs of gelatin supernatant (A and B) and film (C and D) at 50% THF after phase separation.

4.3.3 Fabrication and structural characterization of elastomeric gelatin gel via co-solvent system

From the SEM analysis of gelatin, the structural transformation due to coacervation has been observed. Coacervation in gelatin at 50% THF has formed an microporous film while they form segregated particles at 50% ethanol. At 50% THF, we observed that only a fraction of gelatin has sedimented at the bottom and the rest was present as coacervates in the supernatant solution. The proportion of gelatin coacervates which has sedimented increased with increasing THF proportion as shown in Figure 4.4. At 50% THF, the concentration of gelatin in the supernatant has dropped from 10 mg/ml to ~5 mg/ml due to phase separation and further drops to ~0.02 mg/ml at 80% THF. Film formation starts at 50% THF and >99% of gelatin

coacervates were driven to form film at the bottom of container from 70% THF onwards. We fabricated the gelatin film at 80% THF and at 80% ethanol to compare the difference in the structural transformation. In the latter case, phase separation was random and coacervates aggregates to deposit on the walls of the container and were white in colour, whereas a translucent film has formed at 80% THF as shown in Figure 4.5. The difference in the colour of the films could be due to difference in microstructure of the films and was further characterized by microscopy techniques. In case of 80% ethanol, the phase separated structure was made up of spherical particles, whereas a fused film has formed in case of 80% THF as shown in Figure 4.6. The 80% ethanol film was not able to maintain its structure and got ruptured during handling due to the presence of individual particles and a fewer inter-particle interaction. The particles present in 80% ethanol film were spherical and poly-dispersed with size 400-1400 nm (calculated using IMAGJ software). The gelatin film at 80% THF has microporous structure and has pore size in the range of 10-35 µm as shown in Figure 4.7.

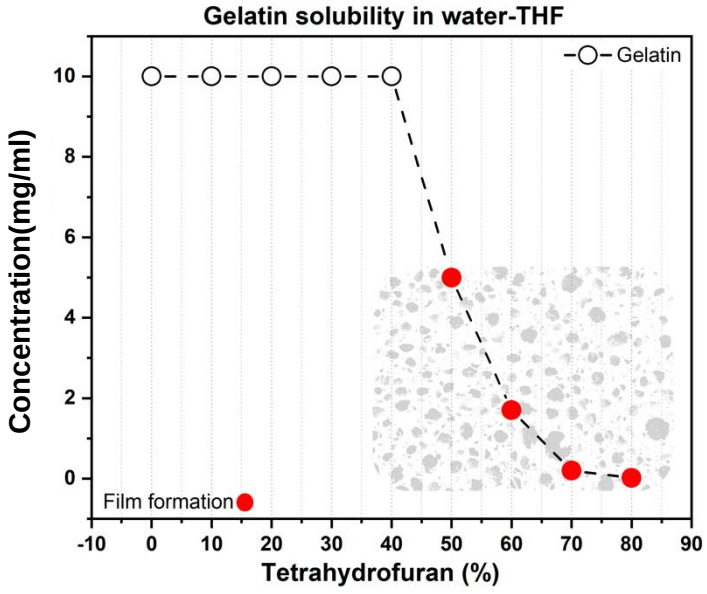

Figure 4.4. Decrease in gelatin concentration in supernatant with increase in THF proportion due to sedimentation of coacervates

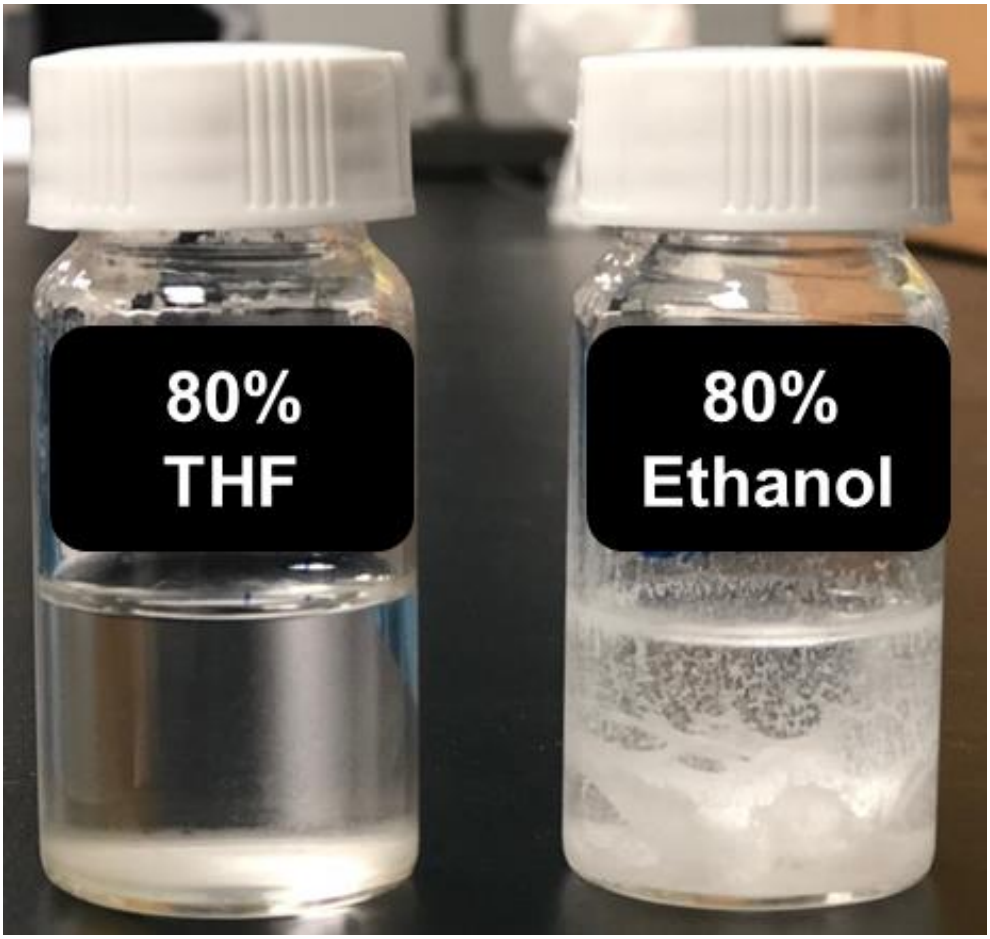

Figure 4.5. Visual analysis of coacervation/phase separation in 80%-THF and 80% ethanol co-solvent system

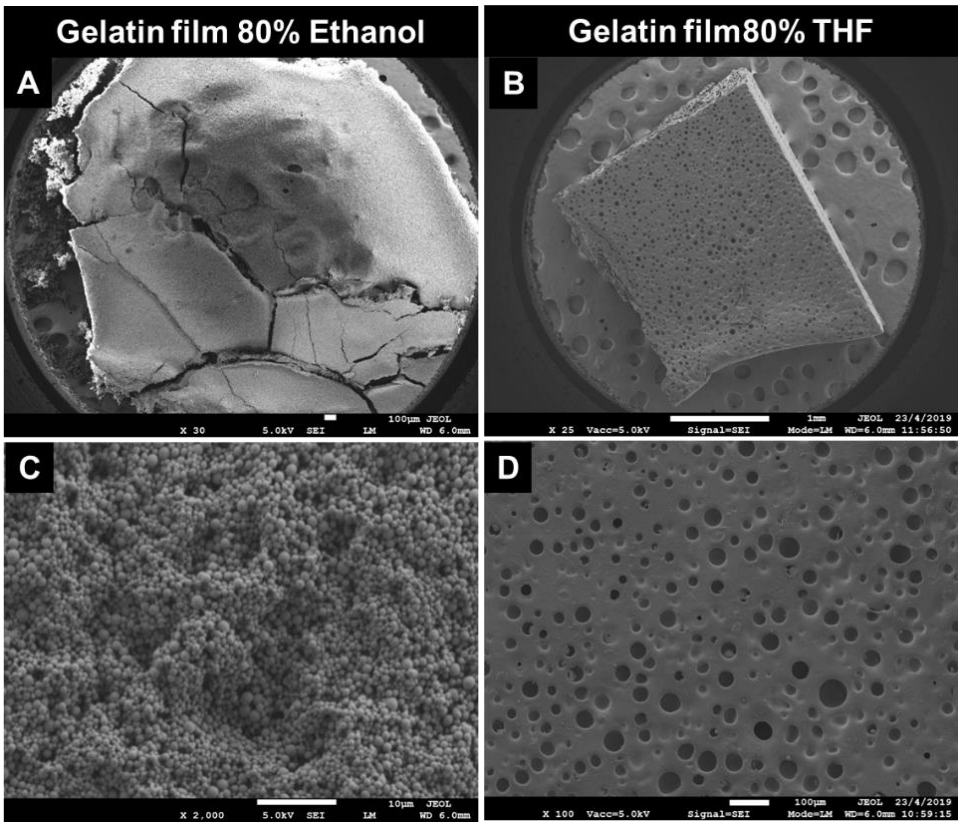

Figure 4.6. SEM micrographs of gelatin film at 80% ethanol (A (30X) and C (2000X) film and 80% THF (B (25X) and D (100X). Film at 80% ethanol have aggregated spherical particles whereas 80% THF film has microporous structure.

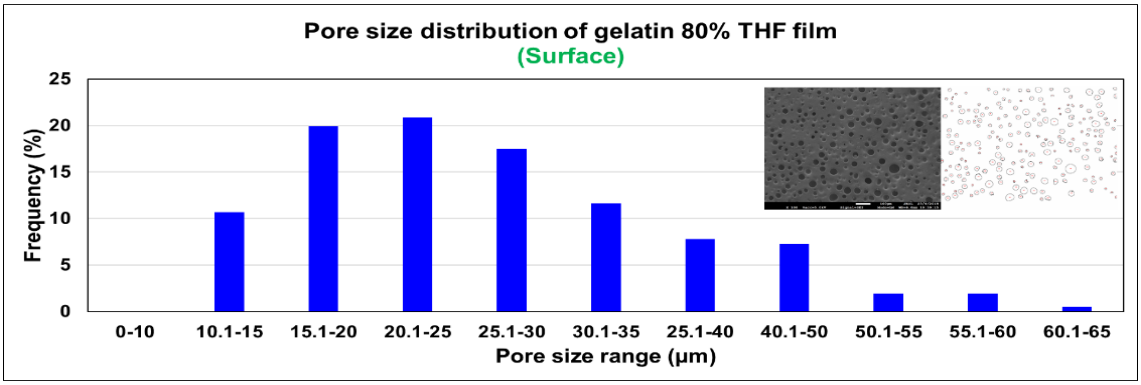

Figure 4.7. Pore size analysis of 80% THF gelatin film in freeze dried state

4.3.4 Elucidation of film fabrication and chemical analysis for gelatin film fabrication in THF as co-solvent

Effect of THF on transmittance and structural transformation in gelatin aqueous solution has been observed in the abovementioned studies. To understand the chemical and structural transformations in the gelatin caused by the THF, the films were further studied by SEM and FTIR spectroscopy. Gelatin solutions with 43.8% THF were prepared, followed by addition of THF to increase the THF proportion from 43.8% to 60% and 80% THF, finally. Samples were freeze dried instantaneously after mixing for 30 seconds to observe the effect of THF on the gelatin microstructure. The samples were then characterized by ATR-FTIR spectroscopy and SEM microscopy. In the SEM images shown in Figure 4.8, small particles were present in 43.8% THF which corresponded to maximum transmittance at 600 nm before phase separation as shown in Figure 4.1. Large spherical micro-sized particles were present in 60% THF as shown in Figure 4.8 B. The large spherical particles could have formed from the aggregation of smaller particles in the liquid phase. It has been reported that the MDP has the potential to grow into larger particles either via nucleation or growth mechanism or through coalescence of droplet phase if the attraction forces are strong enough [21, 24]. A solid film was observed at 80% THF, which has been reported in the section above. To characterize the chemical changes due to the THF, all powder samples were characterized by ATR-FTIR. In the ATR-FTIR spectrum shown in Figure 4.8 D, it was observed that the peak at 1053 cm^{-1} emerges with increase in THF proportion at 60% THF and intensity decreases in 80% THF. Band at 1053 cm^{-1} belongs to C-O stretching. This could be due to change in the structural organization of gelatin backbone due to water-THF co-solvent.

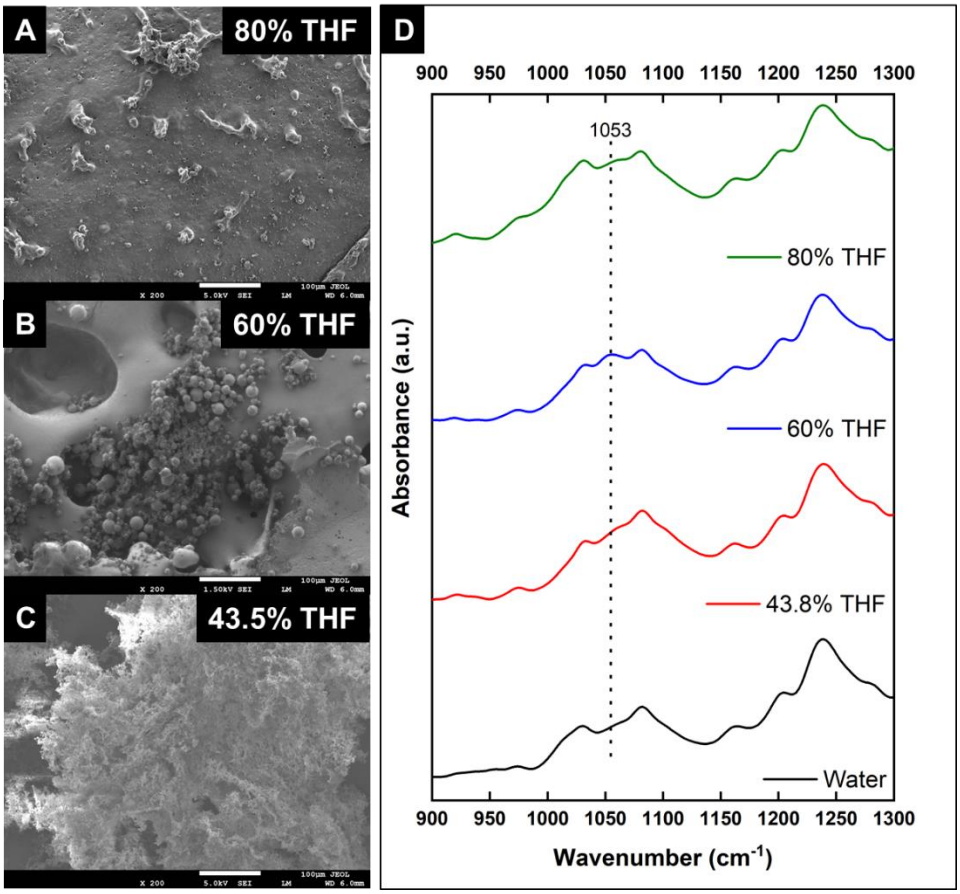

Figure 4.8. SEM images of gelatin at A) 80% THF, B) 60% THF and C) 43.5% THF proportions (10 mg/ml). ATR-FTIR spectrum of gelatin in water, 43.8% THF, 60% THF and 80% THF.

4.3.5 Mechanical properties of stretchable gelatin gel

Gelatin film fabricated at 80% THF was peeled out and tested for the mechanical properties via tensile testing as shown in Figure 4.9. Film fabricated with 80% ethanol was not able to maintain its integrity, whereas film at 80% THF was stable. Gelatin film formed with 80% THF has a Young's modulus of 78.71 ± 0.48 kPa, and 1269±80%, strain at break. Thermal gravimetric analysis of gelatin gel at 80% THF demonstrates the loss of solvents in two different stages. The first loss took place from 0-80^{o}C (27.35%), attributed to THF present in the film and another around 100^{o}C (18.30%), attributed to water.

The gelatin film fabricated at 80% THF has 45.65% of total solvent content (water and THF). To check the stability of gelatin film at 80% THF, film was suspended in distilled water at room temperature for 24 hours. It was observed that the gelatin film swelled up and loses it integrity.

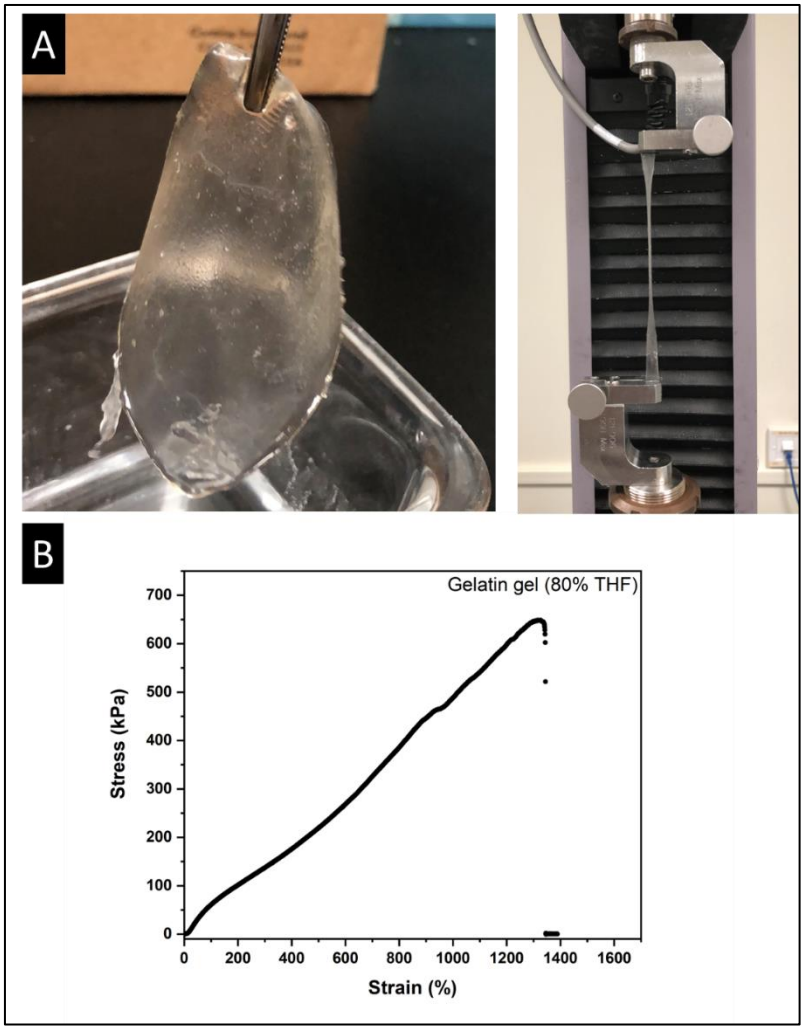

Figure 4.9. Images of gelatin film formed at 80% THF (A) and stress vs strain graph (B) of gelatin film obtained via tensile test on mechanical tester with 50 N load at 80% THF.

4.3 Conclusion

Behaviour of gelatin in co-solvent systems of water-ethanol and water-THF has been studied. THF as co-solvent for gelatin aqueous solution was able to form an elastomeric film, whereas gelatin in ethanol as co-solvent was not able to form a stable film. The elastomeric gelatin formed at 80% THF has a Young's modulus and strain at break of 78.71±0.48 kPa and 1269±80% respectively. However, the film swelled and lost integrity when suspended in distilled water at room temperature. Gelatin gel that is formed through solvophobic interactions (due to THF) does not possess enough integrity to retain its shape and to counterbalance the adsorbed water. Other studies have shown that introduction of covalent crosslinks like glutaraldehyde, methacrylamide, hexamethylene diisocyanate etc., would compromise the stretchability of elastomeric gelatin gel and makes it stiff rather than soft as shown in the literature [31, 32]. Therefore, for subsequent chapters, supramolecular crosslinking will be introduced to enhance the stability of gelatin gel, while maintaining a low modulus (soft gel) that is compliant with soft tissues. The supramolecular crosslinking is introduced via substitution with ureidopyrimidinone as elaborated in the next chapter.

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Chapter 5

Synthesis of ureidopyrimidinone synthon and ureidopyrimidinone substituted gelatin derivatives

Chapter 5 demonstrates the synthesis and chemical characterization of ureidopyrimidinone and ureidopyrimidinone substituted gelatin derivatives with different degrees of Upy substitution. The chapter begins with introduction and material and methods, followed by section 5.3.1 (Results and discussion) which presents the organic synthesis of ureidopyrimidinone and its chemical characterization by FTIR and NMR spectroscopy. Section 5.3.2 (Results and discussion) introduces the synthesis methods of ureidopyrimidinone substituted gelatin derivatives with ties chemical characterization.

5.1 Introduction

Gelatin is a hydrolyzed collagen, manufactured from animal sources like pig skin /bones and bovine hides/bones. Gelatin can be classified into two types based on the hydrolysis process – gelatin A (acid hydrolysis) and gelatin B (alkaline hydrolysis). It has almost similar amino acid composition (shown in Table 5.1) to collagen even though there are some variations due to the synthesis process. Gelatin A has higher proportion of secondary amino groups ($-NH_2$) in comparison to gelatin B due to deamination of glutamine and asparagine to glutamic acid and aspartic acid, respectively during alkaline hydrolysis. The functional groups like amino, hydroxyl and carboxyl groups, possessed by the amino acids including lysine ($-NH_2$), hydroxyproline (-OH), aspartic acid (-COOH) and glutamic acid (-COOH), in gelatin or proteins plays a vital role determining the structural, physical and chemical properties for gelatin hydrogel. These functional groups present in the side chains of amino acids, act as site of functionalization to introduce new properties in the gelatin.

Table 5.1. Amino acid composition of pig skin gelatin [1]

Sno.	Amino acid	g/100g	Sno.	Amino acid	g/100g
1	Alanine	10.7	11	Cysteine	0
2	Glycine	26.4	12	Methionine	0.88
3	Valine	2.77	13	Arginine	9.1
4	Leucine	3.34	14	Histidine	1.01
5	Isoleucine	1.36	15	Lysine	4.14
6	Proline	16.2	16	Ornithine	0
7	Phenylalanine	2.56	17	Aspartic acid	6.7
8	Tyrosine	0.6	18	Glutamic acid	11.3
9	Serine	4.13	19	Hydroxyproline	13.5
10	Threonine	2.19	20	Hydroxylysine	1.04

Gelatin behaves like a low viscosity liquid aqueous solution at physiological temperature and forms a hydrogel below sol-gel transition temperature (20-25^{o}C), which makes the involvement of external crosslinking agent important for its stability under physiological conditions. Several crosslinking agents including chemical and enzymes have been used for stabilizing gelatin hydrogel. Crosslinking of gelatin with chemical and enzymatic agents exploits the amino, hydroxyl and carboxyl groups of gelatin biopolymer as shown in Table 5.2.

Table 5.2. Chemical/enzymatic crosslinking methods and functional groups of gelatin involved in crosslinking

Sno.	Crosslinking agent	Functional group	Reference
1	Methacrylate	-OH, -NH_2	[2]
2	Genipin	-NH_2	[3]
3	Transglutaminase	-NH_2	[4]
4	Glutaraldehyde	-NH_2	[5]
4	Terephthalaldehyde	-NH_2	[6]
5	Thiol Norbornene	-NH_2	[7]
6	Hexamethylene Diisocyanate	-NH_2	[8]
7	Phenyl isothiocyanate	-NH_2	[9]
8	Ureidopyrimidinone	-NH_2	[10]
9	Ionic crosslinking	-COOH	[11]

Apart from covalent crosslinking mentioned above, several non-covalent crosslinking methods agents have been developed by researchers for crosslinking for polymers. Non-covalent crosslinking of polymer involves introduction of hydrophobic interactions, ionic interactions, metal-ion coordinate complexes, host-guest complexes and hydrogen bonding. In comparison to covalent crosslinking, it is complex, dynamic and microenvironment sensitive. Due to the complexity of such interactions, they have not been explored much and require an extensive study to understand the

prerequisite of micro and macro environmental conditions for supramolecular crosslinking in the materials. To mimic the natural supramolecular systems, many molecules have been synthesized to mimic natural materials including cucurbiturils [12], cyclodextrin-adamantane [13] and ureidopyrimidinone [14]. Among these given molecules, ureidopyrimidinone has been widely explored for the synthesis of telechelic polymers and supramolecular crosslinking.

Ureidopyrimidinone is a pyrimidine derivative with dimerization potential developed by Meijer group. It would undergo reversible dimerization via quadrupole hydrogen bonds. It is hydrophobic in nature and possess strong dimerization constant of 10^6 M^{-1} in chloroform [15]. Due to its strong dimerization constant, it has been employed for functionalization to introduce supramolecular crosslinking. Dimerization of ureidopyrimidone takes place in two different patterns: DDAA and DADA (D-Donor, A-Acceptor). The pattern of dimerization depends upon the tautomeric form of ureidopyrimidone. The tautomeric form of ureidopyrimidone relies on the substitution at C-6 of isocytosine ring (A) and the terminal urea group (B). Ureidopyrimidone exist in three different tautomeric forms as shown in Figure 5.1 and are named with respect to its form: A) 6[1H]-pyrimidinone B) 4[1H]-pyrimidinone C) pyrimidin-4-ol. Among the tautomeric forms, 4[1H]-pyrimidinone and pyrimidin-4-ol are able to undergo dimerization. The tautomeric form adopted by the ureidopyrimidone will be controlled by following factors: solvent, temperature and radiation exposure.

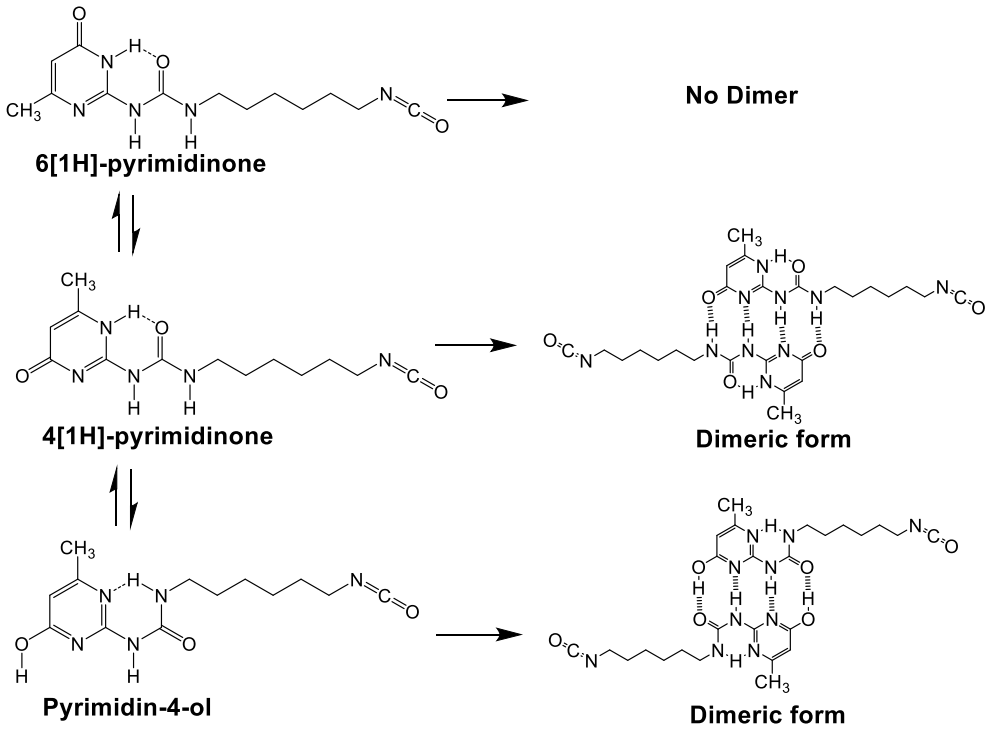

Figure 5.1. Tautomeric forms of Ureidopyrimidinone and their dimeric forms

It is hydrophobic in nature due to its derivation from nitrogenous base and have been exploited for its substitution in hydrophobic polymers. To manipulate this material, it was mixed in organic solvents like toluene, tetrahydrofuran and chloroform for supramolecular crosslinking/ hydrogen bonding. Despite its hydrophobicity, a few researchers have tried to substitute ureidopyrimidinone onto hydrophilic polymers to develop hydrogel systems. Due to the hydrophobic behavior of ureidopyrimidinone, it has introduced non-directional hydrophobic interactions in the host polymer which renders the polymers insoluble in water. The hydrophilic polymers which were initially capable of forming a hydrogel had lost their ability to form a hydrogel after substitution and require extreme acidic or basic condition to make them soluble [10]. In this chapter, we accomplished the organic synthesis of ureidopyrimidinone (Upy) followed by its substitution in gelatin biopolymer. Upy substituted gelatin derivatives with different degrees of Upy substitution has been achieved by varying the Upy feed. Further, the Upy and Upy substituted gelatin derivatives

were characterized for their purity and chemical characterization. The corresponding hydrogel properties from this synthesis will be discussed in the next chapter.

5.2 Experimental Methods

5.2.1 Synthesis of ureidopyrimidinone synthon

A typical procedure from an earlier report was used to synthesize ureidopyrimidinone or 2(6-isocyanatohexylaminocarbonylamino)-6-methyl-4[1H] pyrimidinone. Two grams (0.0159 mol) of 2-amino-4-hydroxyl-6 methyl pyrimidine and 21.5 g (0.128 mol) of 1, 6-diisocyanatohexane was mixed in anhydrous pyridine (70 ml, 0.906 mol) and heated under continuous stirring at $100^{O}C$ for 16 hours in N_2 atmosphere. Further pentane (200 ml) was added to precipitate the white powder, which was further filtered and washed with acetone three times. The obtained white powder was dried in vacuum at $50^{O}C$. Product was vacuum distilled to remove the excess 1, 6-diisocyanatohexane from the product [16].

5.2.2 Synthesis of ureidopyrimidinone substituted gelatin

2 grams of Gelatin A (300 bloom) was dried in vacuum at $60^{o}C$ to remove the moisture and was mixed in an anhydrous 70 ml DMSO at $60^{o}C$ for 48 hours with continuous stirring to get clear gelatin solution. Synthesis of Upy substituted gelatin involves two steps. In the first step, Upy (0.10, 0.30 and 0.50 grams) was added to gelatin solution and was continuously stirred at $60^{o}C$ in N_2 atmosphere for 24 hours. In the second step, dibutyltin dilaureate was added to the mixture as a catalyst and was stirred for another 24 hours at $60^{o}C$ in N_2 atmosphere. After 48 hours of reaction, the solution was kept at room temperature to cool down and was precipitated with isopropanol. The precipitated sample was centrifuged and washed with isopropanol. The pellet

was suspended in water and was freeze dried to obtain the Upy substituted gelatin synthesized by two-step method (GEUPYII).

Table 5.3. Synthesis of Upy substituted gelatin derivatives reaction conditions and reactants

Samples	**Gelatin (mg/ml)**	**Upy (g/2g gelatin)**	**Temperature (ºC)**	**Time (hours)**		**Catalyst (per 2g gelatin)**
				Step I	**Step II**	
GEUPYII (0.10)	28.57	0.10	60	24	24	500 µl
GEUPYII (0.30)	28.57	0.30	60	24	24	500 µl
GEUPYII (0.50)	28.57	0.50	60	24	24	500 µl

5.3 Results and discussions

5.3.1 Synthesis of ureidopyrimidinone synthon and its characterization

Synthesis of ureidopyrimidinone synthon (Upy-synthon) and its substitution in polymers have been carried out through various strategies depending upon the degrees and site of substitution. 2(6-isocyanatohexylaminocarbonylamino)-6-methyl-4[1H] pyrimidinone is the most common form of Upy-synthon, which has been employed for its substitution in hydroxyl (-OH) and amino groups (-NH_2) due to free isocyanate (-NCO) group. It has been synthesized by reacting 6-methylisocytosine (6MC) with hexamethylene diisocyanate (HDI), catalyzed by pyridine in nitrogen atmosphere due to sensitivity of isocyanate group

towards moisture as shown in Figure 5.2 [16]. Substitution reaction involves grafting of HDI over amine (-NH_2) of 6-methylcytosine, leaving the other isocyanate group free for its substitution on host polymer. 2(6-isocyanatohexylaminocarbonylamino)-6-methyl-4[1H] pyrimidinone has been employed for the substitution of Upy over hydroxyl/amino group in polymers like polybutadiene [17], cellulose [18], poly vinyl alcohol [19], dextran [20], polyethylene glycol (PEG) [21], polycaprolactone-diol (PCL-diol) [21] and gelatin [10]. Both Upy substituted telechelic and side chain functionalized polymers has been synthesized using the 2(6-isocyanatohexylaminocarbonylamino)-6-methyl-4[1H] pyrimidinone. This Upy-synthon was synthesized as white powder and was characterized by FT-IR and NMR spectroscopy to ensure the purity of the Upy-synthon. In FTIR spectrum of ureidopyrimidinone shown in Figure 5.3, transmittance at 3216 cm^{-1} and 3144 cm^{-1} corresponds to N-H groups involved in dimerization [22]. Presence of these two peaks confirms the successful synthesis of Upy-synthon and their dimerization. Transmittance at 2279 cm^{-1} and 1701 cm^{-1} represents the isocyanate group (-NCO) and isocytosine carbonyl group (C=O) of Upy-synthon [17, 23]. Isocyanate peaks represents unreacted isocyanate group of hexamethylene diisocyanate (HDI), available for grafting on target polymer or molecule. In addition, 1669 cm^{-1} corresponds to urea carbonyl group of Upy-synthon which confirms the grafting of HDI on isocytosine ring as shown in Figure 5.3 [24]. To further ensure the purity of Upy-synthon, Upy-synthon powder was characterized by ^{1}H NMR spectroscopy. In the ^{1}H NMR spectrum of Upy (shown in Figure 5.4), chemical shifts at A (13.1523 ppm), B (11.8986 ppm) and C (10.2159 ppm) belongs represents intermolecular hydrogen bonds formed by the amine groups, whereas chemical shift D (5.862 ppm) represents the intermolecular hydrogen bonding [25, 26]. Their presence in the spectrum indicated the successful synthesis as well as purity of Upy-synthon [22, 27-29]. Conclusively, Upy-synthon has been synthesized successfully and FTIR and NMR analysis provides confirmation of the chemical structure.

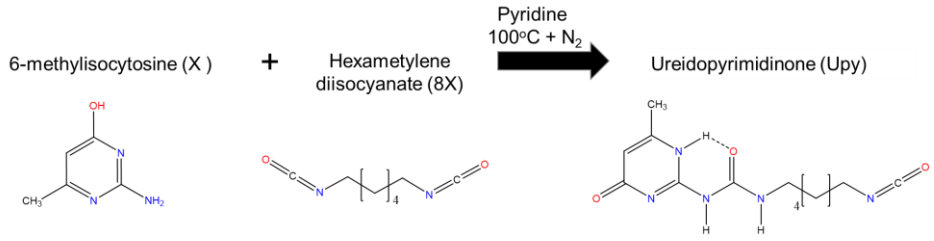

Figure 5.2. Schematics to demonstrate the synthesis of ureidopyrimidinone

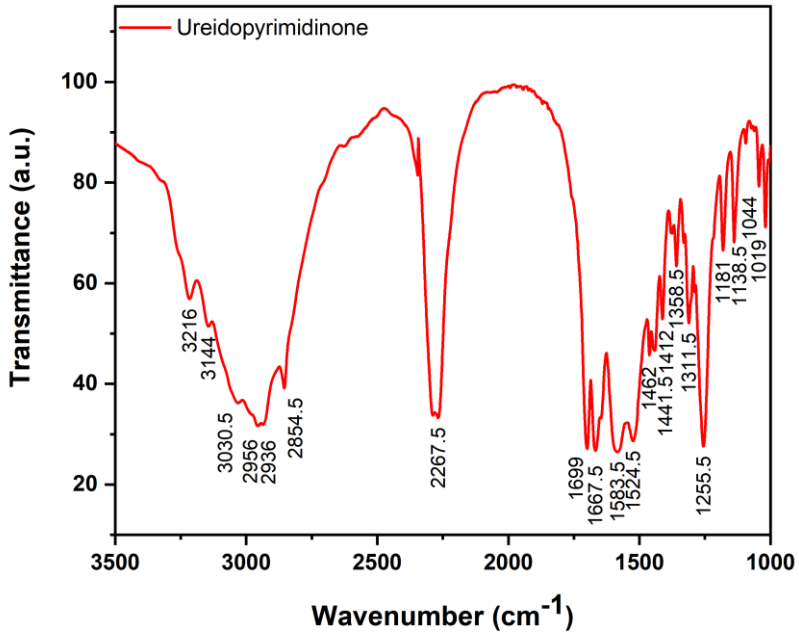

Figure 5.3. FTIR spectrum of Ureidopyrimidinone

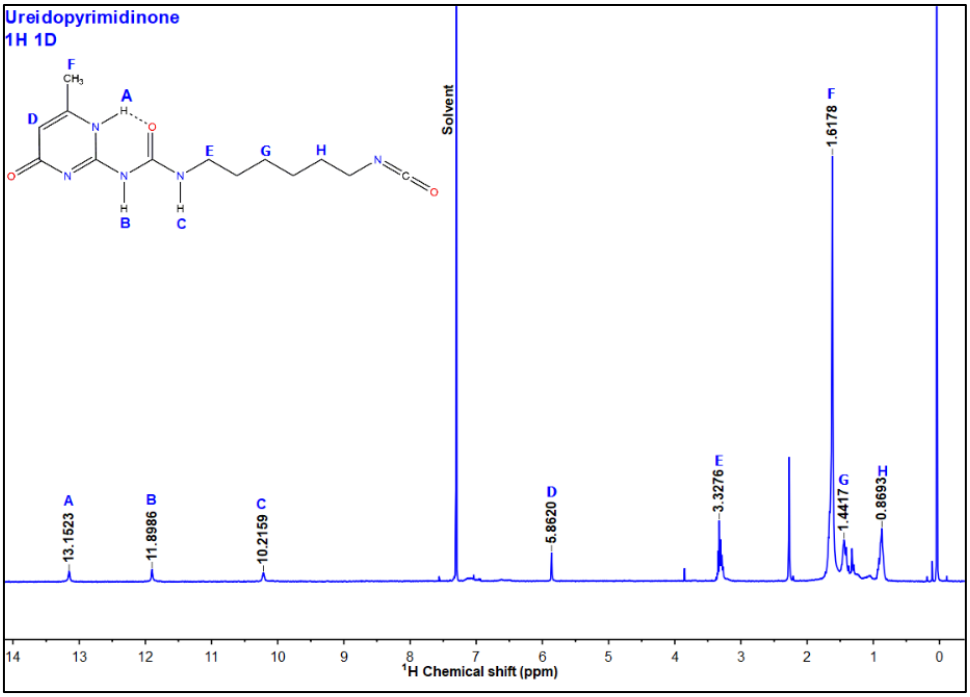

Figure 5.4. ^{1}H NMR spectrum of ureidopyrimidinone (Upy-synthon)

5.3.2 Synthesis of ureidopyrimidinone substituted gelatin derivatives and its characterization

Gelatin biopolymer constitutes of various amino acid residues and in different proportion as shown in Table 5.1. Every amino acid residue varies with each other due to different side chains. The functional groups present on the side chains of amino acid residues would facilitate the substitution of crosslinking agents. Substitution of Upy has been carried in various ways depending upon the chemical functional groups present in the host polymer. In gelatin, primary amino groups (-NH_2) are present in side chain of lysine and hydroxyl groups (-OH) are present in side chain of hydroxyproline, would facilitate the substitution of Upy. Substitution of Upy was carried out in two steps depending on the presence of catalyst. In the first step, gelatin in DMSO was mixed with Upy-synthon to react with amine groups of gelatin biopolymer for 24 hours in N_2 atmosphere at 60^{o}C. Isocyanate group of Upy-synthon reacts faster with

amine groups of gelatin in comparison to hydroxyl groups and does not require a catalyst [24]. Isocyanate group of Upy synthon bears sequence of double bonds as R-N=C=O. The reactivity of isocyanate group is controlled by the positive character of the carbon atom and is susceptible to be attacked by nucleophiles, and electrophilic attack on oxygen and nitrogen [30]. Reaction of isocyanate-amine would result in the formation of urea bond between Upy and gelatin biopolymer as shown in schematics in schematics in Figure 5.5.

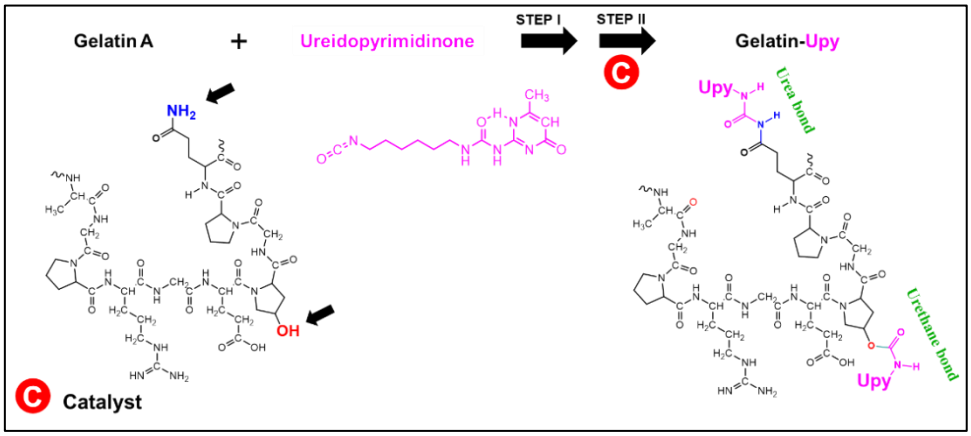

Figure 5.5. Schematics for synthesis of Upy substituted gelatin derivatives via two step method

In the second step, catalyst was injected in the reaction mixture and was continuously stirred at 60°C for next 24 hours. Presence of catalyst would enhance isocyanate-hydroxyl reaction in the second step. Reaction of isocyanates with hydroxyl groups requires catalysts to enhance rate of the reaction. Phenols (calcium, magnesium, strontium, barium, salts of hexanoic, octanoic, naphthenic, linoleic acid), alkali metal salts of carboxylic acids, aliphatic amine, aromatic amines (e.g. diaminobicyclooctane - DABCO) and organometallic compounds (e.g. dibutyltin dilaureate (DBTDL), dibutyltin diacetate) have been used to catalyze the reaction [31]. For substitution of Upy-synthon at hydroxyl groups of gelatin biopolymer, dibutyltin dilaureate was used due to its low toxicity and volatility over other catalysts. DBTDL catalyze

the isocyanate-hydroxyl reaction by forming complex with isocyanate and hydroxyl groups. Electron rich oxygen of isocyanate and hydroxyl groups forms an intermediate complex with positive metal center. Intermediate complex would undergo rearrangement to form urethane bonds. Hou et al. has used the DBTDL for the substitution of Upy over hydroxyl groups of dextran [20]. Presence of DBTDL would preferably boost up the reaction of isocyanates with hydroxyl groups due to higher nucleophilicity of hydroxyl oxygen [32].

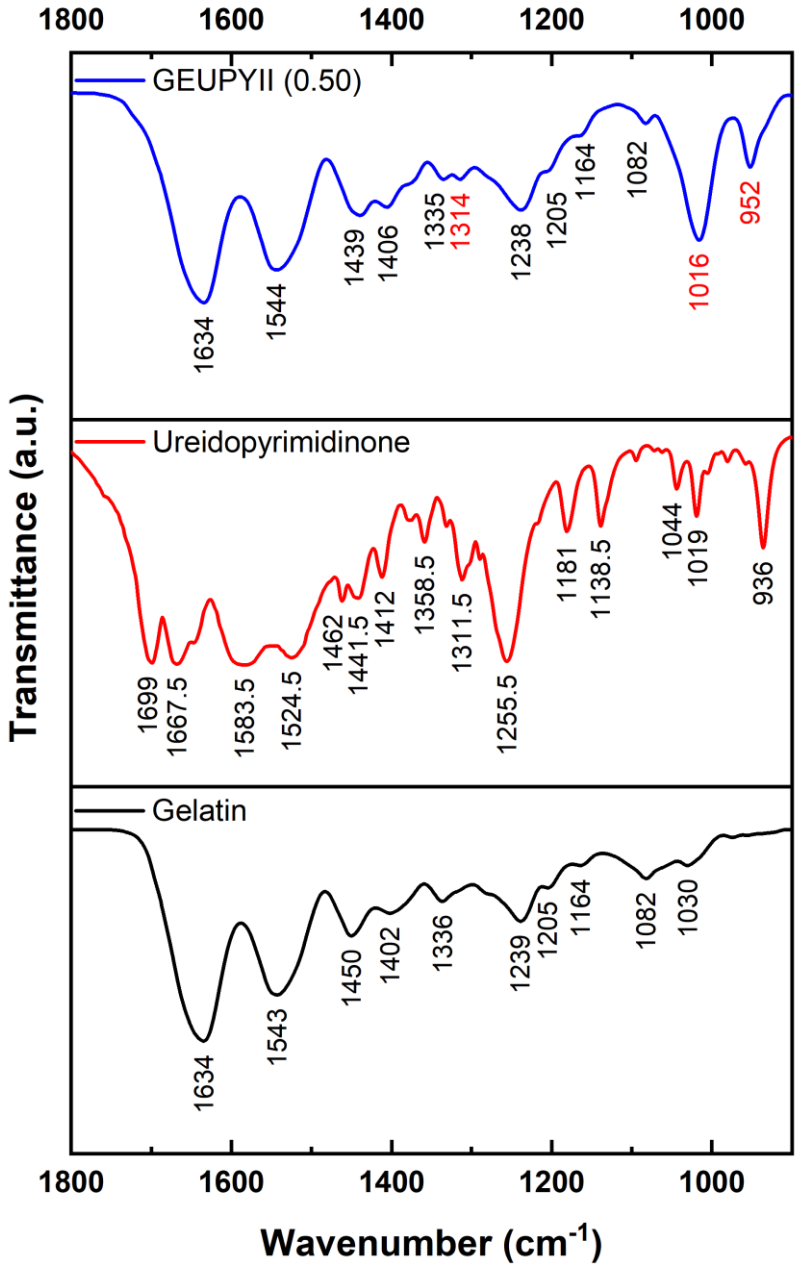

Figure 5.6. FTIR spectrum of GEUPYII (0.50), Upy-synthon and gelatin

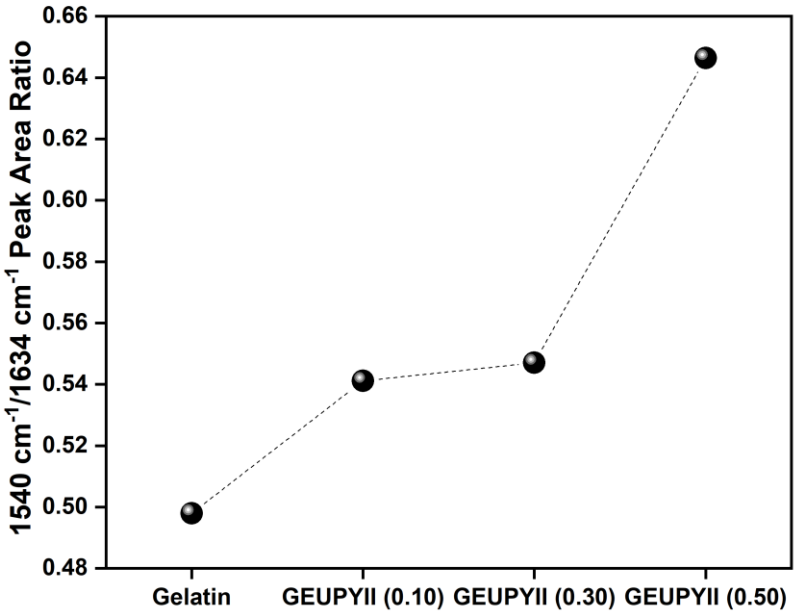

Figure 5.7. Peak area ratio of 1544/1634 cm^{-1} in Upy substituted gelatin derivatives with varying Upy feed

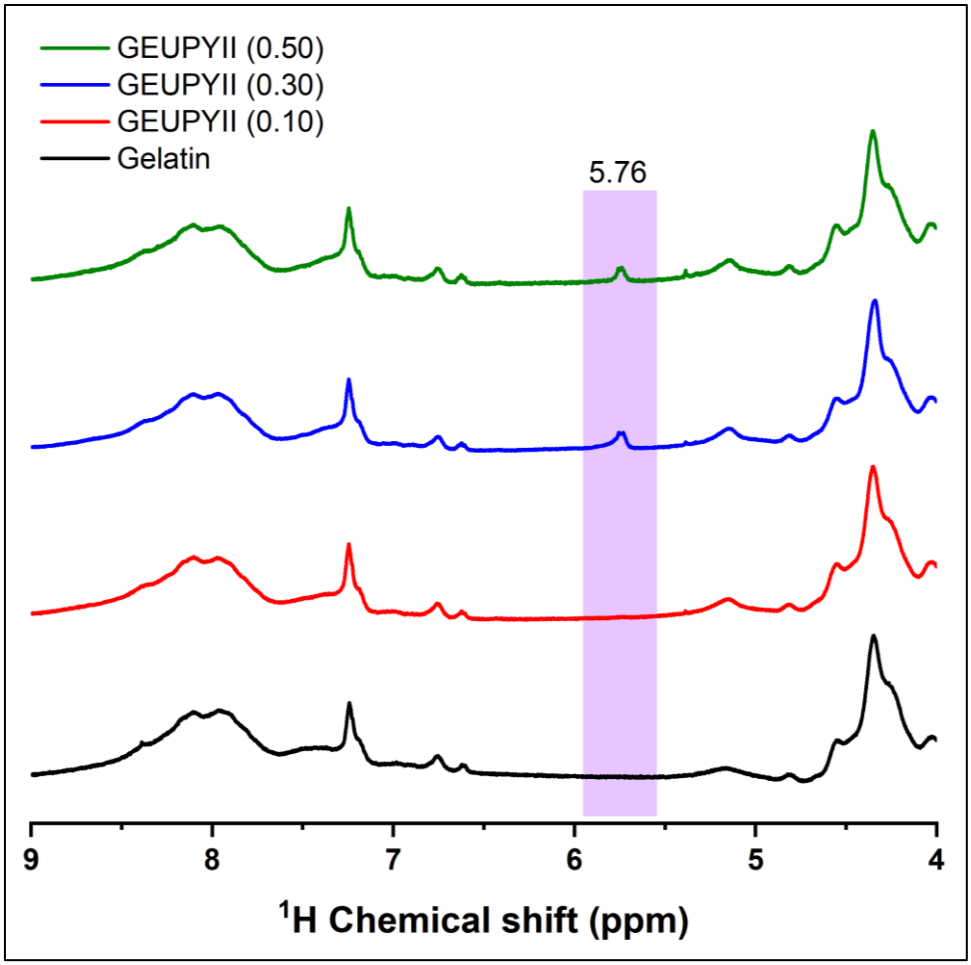

Figure 5.8. ^{1}H NMR spectrum of gelatin and Upy substituted gelatin derivatives (GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50)

Upy substituted gelatin biopolymers with different degrees of Upy substitution was carried out by varying the Upy-synthon feed (0.10, 0.30 and 0.50 g/2g gelatin) in the reaction as shown in Table 5.3. After substitution reaction, the powder samples were precipitated and freeze dried for further studies. Powder samples obtained were characterized by ATR-FTIR and NMR for chemical characterization. The ATR-FTIR spectrum of gelatin and Upy substituted gelatin demonstrates the spectral changes occurred due to substitution of Upy in gelatin. In the spectrum shown in Figure 5.6, transmittance at 1634 cm^{-1} and 1544 cm^{-1} corresponds to amide I (stretching vibrations of C=O bond) and

amide II (bending vibrations of N-H bond) groups. We observed an increase in the peak area ratio of 1544/1634 with increase in Upy-synthon feed in the reaction, which could be due to higher Upy substitution (Figure 5.7). Qualitatively, the presence of peaks in the fingerprint region in ATR-FTIR spectrum of GEUPYII (0.50) in Figure 5.7, including 953 cm^{-1} and 1016 cm^{-1} also confirms the Upy substitution in gelatin. The peak broadening was observed in comparison to Upy peaks could be due to presence of enol tautomer in addition to keto of Upy [33]. In case of Upy substituted gelatin derivatives, the substitution has been confirmed by ^{1}H NMR spectroscopy. The presence of 5.76 ppm chemical shift in ^{1}H NMR spectrum (Figure 5.8) of Upy substituted gelatin derivatives confirm the substitution of Upy on gelatin backbone utilizing its functional groups. As the substitution of Upy is random, it is difficult to quantify the degree of substitution. Increase in intensity of chemical shift at 5.76 ppm with increase in Upy feed to the reaction confirms the increase in degrees of Upy substitution on gelatin biopolymer.

5.4 Conclusions

Synthesis of ureidopyrimidinone and its substitution in gelatin biopolymer has been successfully carried out. Upy substituted gelatin derivatives with different degrees of Upy substitution has been synthesized by varying the Upy-synthon feed in the reaction. In the transmittance studies we observed the highest coacervation in case of GEUPYII (0.50), which is due to higher substitution of Upy (shown by NMR spectrum). Higher substitution is required for the supramolecular crosslinking to stabilize the hydrogel. Therefore, GEUPYII (0.50) was chosen to fabricate a stable elastomeric hydrogel in the next chapter.

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Chapter 6

Fabrication of stretchable GEUPYII hydrogel via co-solvent system

Chapter 6 demonstrated the optimization of co-solvent system for ureidopyrimidinone substituted gelatin derivatives (GEUPYII), studies of co-solvent induced structural transformation studies, elastomeric film fabrication, mechanical, structural and chemical characterization of elastomeric films and elucidation of film fabrication co-solvent method. Section 6.3.1 (Results and discussions) describes the solubility analysis of gelatin and Upy substituted gelatin derivatives and the structural characterization in section 6.3.2 (Results and discussions). Section 6.3.3 (Results and discussions) introduces the elastomeric film fabrication method. Film structural and mechanical properties are explained in section 6.4.4 and 6.4.5. Elucidation of film fabrication studies with its chemical characterization are presents in section 6.5.6

6.1 Introduction

Hydrogel research has been carried out for decades. Hydrophilic polymers have been utilized for the fabrication of hydrogels and can be classified into two main categories based on solubility in water: water soluble hydrophilic polymers (WSHP) and water insoluble hydrophilic polymers (WIHP). Hydrogel fabrication has been carried out either by physical crosslinking or by covalent crosslinking in aqueous environment. Covalent crosslinking has been carried out either by functionalization of the polymer chain with molecules which can be cross-linked by external stimulus or by direct introduction of crosslinking agent (catalyst/enzyme), whereas physical crosslinking has been introduced either by functionalization of the polymer chain with self-assembled molecules which are able to form non-covalent crosslinking by external stimulus like temperature, pH or by addition of external crosslinking agent like metal ions, self-assembled moieties etc. WSHP includes gelatin [1, 2], alginate [3], dextran [4], hyaluronic acid [5], poly (ethylene glycol) [6], polyacrylamide (PAM) [7], poly (ethylenimine) (PEI) [8], poly (acrylic acid) (PAA) [9], poly (vinyl alcohol) [10], poly(vinylpyrrolidone) (PVP) [11]. WIHP polymers include cellulose [12], lignin [13], Poly(N-isopropylacrylamide) (PNIPAM) [14], block copolymers with hydrophobic segments [15], hydrophobically modified polymers (which lost their water solubility or dispersibility) [16]. Hydrogel fabrication process for WIHP has been carried out by employing conventional methods like solvent casting and hot melt processing. In case of solvent casting, WIHP polymers were dissolved in organic/inorganic solvents followed by evaporation to get a dry film, whereas hot melt processing requires heating of the polymer chain above melting temperature to get a desired shape for hydrogel fabrication [17].

Hydrogel fabrication methods like solvent casting and hot melt processing, which have been used for WIHP conventionally are not efficient in generating

the supramolecular interactions for supramolecular hydrogel fabrication and is dependent upon the properties of host polymer. Supramolecular interactions are sensitive to environmental conditions like solvent, tautomerization, temperature, pH, ionic strength etc. and requires a specific set of condition to be fulfilled to form crosslinks. This is especially so for protein-based polymers (PBB) which do not have a melting point and would undergo direct degradation when heated and thus could not be processed by conventional methods like hot melt processing

In PBB, solvent casting has been commonly used for film fabrication. It requires dissolution of PBB in a suitable solvent, which segregates the polymeric chains by intermolecular interactions between solvent and polymer molecules followed by evaporation. However, the substitution of functional groups may decrease the solubility of PBB in their respective solvents which would limit the dissolution of PBB. The limited dissolution of substituted PBB would hamper the hydrogel fabrication via solvent casting and requires new improved systems to fabricate hydrogel films.

Gelatin is a protein-based biopolymer (PBB), which has been modified with various functional groups and have been fabricated into hydrogel by suspending gelatin in distilled water followed by the crosslinking (physical or covalent). However, hydrophobically modified gelatin has been reported to form aggregates and micellar structures in aqueous solution [18, 19]. Aggregates prevent the formation of network required for gel formation in the aqueous environment. To address this issue, researchers have devised various strategies. Feng et al. have used the solvent exchange method to fabricate a stretchable hydrogel which involves the solvent exchange of dimethyl sulphoxide with water of reaction mixture (adamantly/phenyl modified gelation and unreacted adamantly/phenyl derivatives) in dialysis tube to form a hydrogel. The resultant hydrogels have 42 and 34 stretch ratio (stretch ratio: stretched length/original length) for phenyl and adamantyl modified gelatin,

respectively. However, the substituted phenyl and adamantyl were not able to provide sufficient hydrophobic interactions to strengthen the gel and requires the unreacted hydrophobic derivatives present in the reaction mixture to make strong hydrophobic domains to form a stable stretchable hydrogel [20]. Also, the limited solubility of gelatin in DMSO prevents gel fabrication at high gelatin concentrations.

In case of ureidopyrimidinone (Upy) substituted in water soluble PBB, hydrophobic interactions in addition to hydrogen bonding becomes active due to hydrophobic nature of Upy. Hydrophobic interactions are non-specific interactions and act to form spontaneously in presence of water, whereas hydrogen bonding is a directional specific interaction and requires a specific orientation to form in a solution. Upy modified gelatin has been reported to lose its water solubility due to hydrophobic aggregation by Upy groups. It was dissolved in formic acid for hydrogel fabrication by solvent casting. The resultant hydrogel has limited stretchability with 12.58%, strain at break [21]. This limited stretchability could be due to lower substitution or due to lack of Upy dimers required for energy dissipation in stretchable hydrogels. Lack of Upy dimer formation could be due to use of formic acid in solvent casting, as formic acid has been reported to break Upy dimers and prevent further dimerization [22].

To address the dissolution of hydrophobically modified polymers, co-solvent systems have been used to prevent the aggregation. Several solution-based techniques like electro-spinning also require complete dissolution of polymers. Meijer et al. has used 90% tetrahydrofuran (90:10, water: Tetrahydrofuran (THF) to form a solution of Upy modified peptide solution for fabrication of electro-spun fibers [23]. In another study by same group have used 20% methanol to fabricate hydrogel film of polyethylene glycol-Upy (PEG-Upy-PEG) through solvent casting, yielding a material that has 1200% strain at break [24]. In this work, we studied the effect of co-solvent system (water-

tetrahydrofuran) on structural transformations of gelatin and Upy substituted gelatin derivatives (GEUPYII). Substitution of Upy in gelatin would introduce supramolecular interactions like hydrogen bonding and hydrophobic interactions to stabilize the gelatin hydrogel.

6.2 Experimental methods

6.2.1 Solubility of GEUPYII in water and water-THF co-solvent system

For concentration dependent studies, gelatin and GEUPYII derivatives were dissolved in aqueous solution at 0.1, 1.0 and 10 mg/ml and mixed at 60^{o}C for 6 hours. Further, the transmittance of the samples was measured at 600 nm. For *IN SITU* studies, 20 mg of GEUPYII was mixed in 1 ml distilled water at 60^{o}C for 6 hours with continuous stirring to get a clear solution. To study the coacervation, 100 μL aliquots of co-solvent THF was added to the aqueous solution of gelatin stepwise till cloud point. After every step, solution was mixed for 5 minutes to stabilize and the transmittance of the solution was measured at 600 nm using UV-VIS spectrophotometer.

6.2.2 Structural analysis of GEUPYII in water-THF co-solvent system

20 mg of GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50) was mixed in 1ml distilled water at 60^{o}C for 6 hours to get a suspension. THF was added in with respect to the initial volume to make 43.8% THF solution and was mixed together to form a clear solution. Further the THF proportion of the samples was adjusted to 50% THF and incubated at 37^{o}C for 24 hours (final concentration – 10mg/ml).

6.2.3 Fabrication of elastomeric gel

Among Upy substituted gelatin derivatives, GEUPYII (0.50) was chosen due to highest Upy substitution to strengthen the hydrogel with supramolecular interactions. 1g of GEUPYII (0.50) was mixed in 20 ml distilled water at 60^{o}C to make a homogeneous suspension for 6 hours. 20 ml THF was added to the solution to make it 50% THF and continuously stirred at room temperature to form a stable clear solution. Further, the THF was adjusted to 80% proportion by slow addition of 60 ml THF and was incubated overnight at 37^{o}C to form a film (final concentration – 10mg/ml). After incubation, the solution turned from milky to clear solution and the film was peeled off from the bottom of the container for mechanical and chemical analysis.

6.2.4 Elucidation of elastomeric gel fabrication

20 mg of GEUPYII (0.50) was mixed in 1ml distilled water at 60^{o}C for 6 hours with continuous stirring to make a homogeneous suspension. 1 ml THF was added to make the THF proportion to 50%. Further, THF was added to the sample to make it 52.5%, 60% and 70% THF. After THF addition, samples were mixed with pipette for 30 seconds and was stored at -80^{o}C for freeze drying. Freeze-dried samples were observed using scanning electron microscopy to study structural characteristics.

6.2.5 Methods to remove co-solvent water-THF from the elastomeric gel of GEUPYII (0.50)

6.2.5.1 Solvent exchange method

In this method, GEUPYII (0.50) film fabricated at 80% THF was suspended in distilled water for 72 hours. Distilled water was replaced with fresh water after every 24 hours.

6.2.5.2 Rehydration of air-dried film

GEUPYII (0.50) film fabricated at 80% THF was kept in vacuum oven at 37^{o}C for 48 hours to remove the solvent from the films. Further, the air-dried film was kept in distilled water for rehydration for 24 hours. After rehydration, film was studied for tis mechanical properties.

6.2.6 Tensile testing of gelatin films

Elastomeric films were characterized for their mechanical properties using MTS mechanical tester Criterion Model 42. Films were cut into standard dumbbell shaped films using cutter. The samples have a gauge length of 9.53 mm and width at gauge length of 3.18 mm. After sample preparation, thickness of the samples was measured using micro-meter screw gauge. Further, samples were loaded onto the tensile fixtures with 50 N load and tested at a cross-head speed of 1 mm/sec.

6.2.7 Swelling analysis of air-dried gelatin and GEUPYII (0.50) film

Air-dried gelatin and GEUPYII (0.50) films fabricated at 80% THF were weighed and were suspended in distilled water for swelling analysis. Swelling of films were observed by weighing the swelled films after tapping on tissue paper at different time interval till 48 hours.

6.3 Results and discussion

6.3.1 Solubility analysis of GEUPYII in water and co-solvent system

Gelatin, GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50) freeze-dried samples were suspended in water in different concentrations (0.10, 1.00 and 10 mg/ml) and was incubated at 60^{o}C for 24 hours to achieve a homogeneous suspension. After 24 hours of incubation, turbidity of samples was measured through transmission test at 600 nm in a microplate reader. Gelatin sample has more than ~90% transmittance at all concentrations, whereas GEUPYII samples showed a significant increase in turbidity with increase in concentration. However, gelatin and GEUPYII samples have almost similar transmittance at 0.1 mg/ml, turbidity increases with increase in concentration of GEUPYII samples as well as increase in Upy substitution (shown in Figure 6.1). This demonstrates that the formation of larger aggregates is due to increase in concentration as well as Upy substitution. Among GEUPYII samples, GEUPYII (0.50) had the highest turbidity at 1 and 10mg/ml and a significant increase in turbidity was also observed at 10 mg/ml.

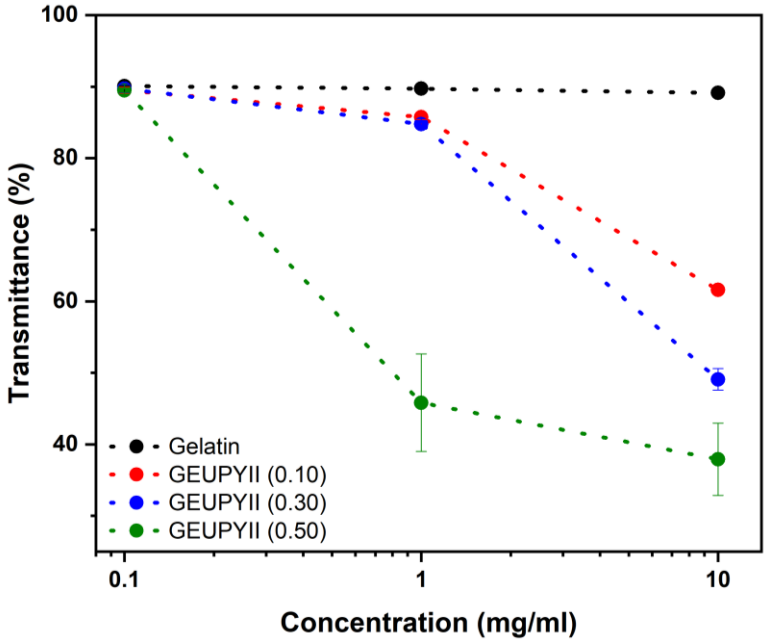

Figure 6.1 – Concentration dependent transmission analysis of gelatin and Upy substituted gelatin derivatives (GEUPYII).

From the concentration dependent studies of gelatin and GEUPYII samples, an increase in turbidity was observed with increase in concentration and Upy substitution due to limited solubility. To enhance the dissolution of polymers, gelatin and GEUPYII samples were exposed to different proportion of water-THF system. To study the effect of THF, aqueous solutions were prepared with 20 mg/ml concentration and THF was added step wise to the aqueous solutions. After addition of THF, transmission of samples after each step was measured at 600 nm. The addition of THF would decrease the polymer concentration as well as increase the THF proportion in the samples. For control, aqueous samples were diluted with water instead of THF for comparison. In situ transmission test for gelatin and GEUPYII samples are shown in Figure 6.2. In case of gelatin, dilution of samples with THF remained stable with >90% transmission till ~43.8% THF and phase separation took place with further increase in the THF proportion to ~47% THF. This demonstrate the stability limit of gelatin in water-THF co-solvent system till 43.8% THF and the point

of phase separation. However, transmission of all GEUPYII samples increases with increase in THF proportion. For GEUPYII (0.10) and GEUPYII (0.30), transmission of the solution first increases till 43.8% THF and undergo a phase separation with further increase in THF proportion. The point of phase separation for GEUPYII (0.10) and GEUPYII (0.30) is simliar to gelatin. Transmission of GEUPYII (0.50) solution increases till 50% THF and phase separation takes place after further increase in THF proportion to 52.5% (not shown in graph). Increase in transmission of GEUPYII samples demonstrates the decrease in size of aggregates with increase in THF proportion and this could be due to decrease in inter and intra molecular interactions, which has segregated the aggregates.

Upy is a self-dimerization hydrophobic moiety and has been used for its substitution in hydrophobic polymers. Substitution of Upy in hydrophilic polymers has been known to introduce hydrophobic effects, which will cause aggregation in hydrophilic polymers. In Upy substituted gelatin derivatives (GEUPYII), aggregates formation was observed which has increased the turbidity of solution. A significant change in turbidity was observed when the concentration was increased from 1 mg/ml to 10 mg/ml. ΔG_{HI} (Gibbs free energy for hydrophobic interactions) decreases linearly with increase in concentration of hydrophobic groups. Increase in concentration of GEUPYII and Upy substitution in GEUPYII cause decrease in ΔG_{HI} [25]. Due to hydrophobic aggregation and intrachain crosslinking, GEUPYII samples lost their ability to form a network, which is requisite for hydrogel fabrication. Studies reported by Shokrollahi and coworkers have reported the issue of solubility and inability to form hydrogel in aqueous environment for Upy substituted gelatin derivatives [26].

Gelatin behaves as a hydrocolloid due to its composition. It has a tripeptide repeat of Glycine-X-Y, where X is usually proline and Y is hydroxyproline. The tripeptide fragment of gelatin chain is hydrophobic when X is proline and

Y is hydroxyproline and is interspersed between hydrophilic fragments. The stability of gelatin in aqueous solution is due to the presence of charged amino acid like lysine, glutamic acid and aspartic acid etc. During synthesis of GEUPYII, Upy has been substituted over amino and hydroxyl groups in the gelatin. Substitution of Upy in gelatin has destabilized the charge and enhance the hydrophobicity. Due to tripeptide and Upy, both gelatin and GEUPYII can be assumed to behave like a random block copolymer with hydrophilic and hydrophobic fragments.

Co-solvent systems have been used to fabricate micellar structures/ polymerosome of block copolymers. Selection of co-solvent plays a vital role in formation of micellar structures and should be chosen based on the solubility of hydrophobic/hydrophilic fragments. In case of GEUPYII, Upy solubility has been studied extensively in dimethyl sulfoxide, toluene, tetrahydrofuran and chloroform. Among the given solvents, dimethyl sulfoxide and tetrahydrofuran have miscibility in water and can act as co-solvent. However, Upy has been reported to exist in 6[1H]-pyrimidinone tautomer in dimethyl sulfoxide, which cannot undergo self-dimerization [27]. Based on the given reports, tetrahydrofuran was selected as a co-solvent in which Upy exist in pyrimidin-4-ol form and can undergo self-dimerization. To understand the effect of co-solvent system, gelatin and GEUPYII was exposed to different proportion of water-THF *in situ*. To examine the effect of THF proportion in water-THF co-solvent system, transmission of gelatin and GEUPYII solution at 600 nm was measured. In case of turbid aqueous solutions of GEUPYII, transmission increases with increase in THF proportion. Transmission of GEUPYII (0.10) and GEUPYII (0.30) increases till 43.8% THF and decreases with further increase in THF proportion due to coacervation as shown in schematics shown in Figure 6.3. However, transmission of GEUPYII (0.50) increases till 50% THF and becomes turbid with further increase of THF to 52.5% THF. Decrease in turbidity or increase in transmission till 43.8% THF demonstrates a decrease in inter and intra molecular interactions and the transformation of a collapsed

state to extended state as demonstrated in schematics section B transition II'/III' of Figure 6.3. It is inferred from the earlier studies that addition of THF would decrease the intra and inter molecular interaction. Stephen et al. has reported the decrease in hydrophobic interaction by addition of THF as co-solvent in phase separated aqueous solution of polydimethylsiloxane [28].

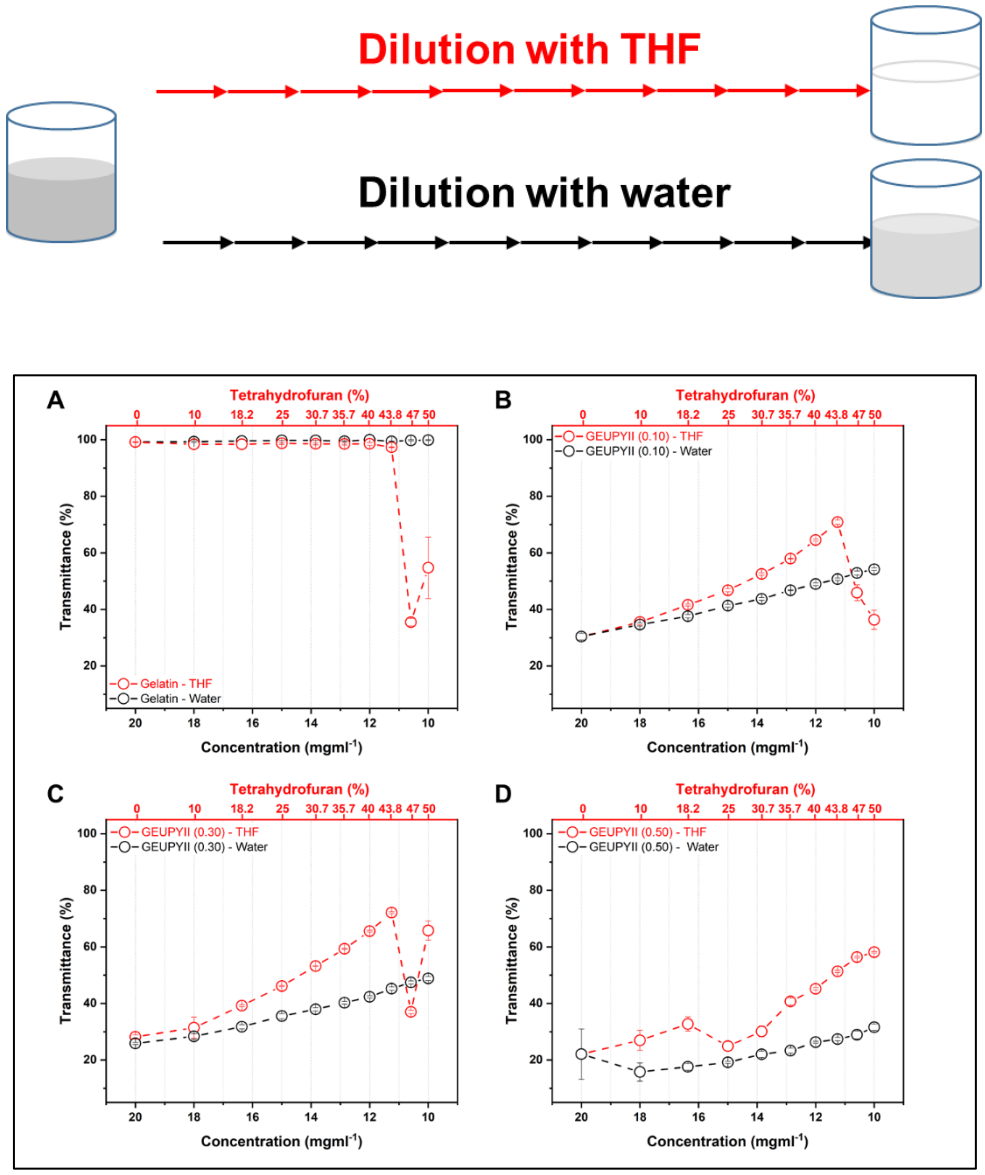

Figure 6.2. *In situ* transmission analysis of A) gelatin, B) GEUPYII (0.10), C) GEUPYII (0.30) and GEUPYII (0.50) samples by diluting with Water and THF

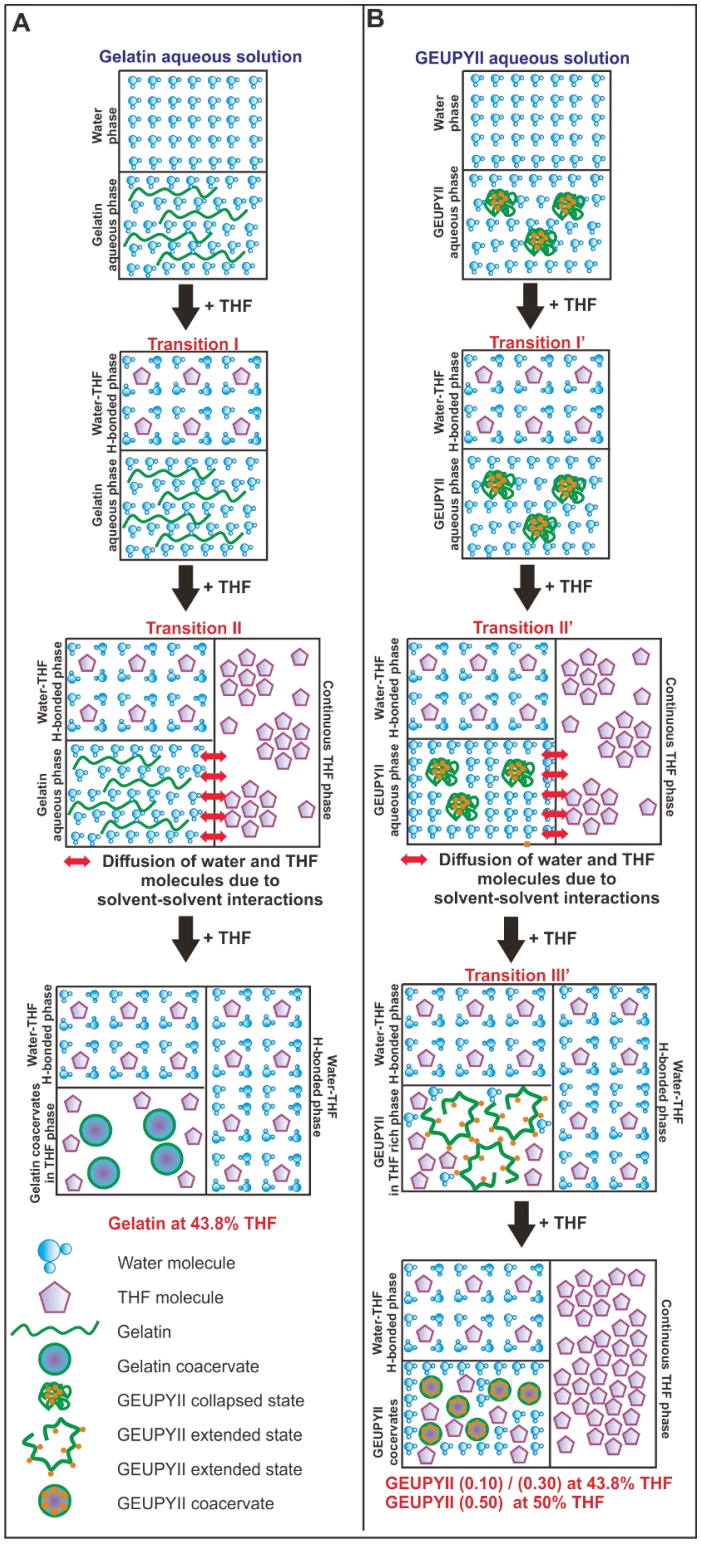

Figure 6.3. Schematics to demonstrate the coacervation of A) gelatin and B) GEUPY in water-THF so-solvent system

Gelatin solution in water-THF co-solvent system remains stable till 43.8% THF and undergone coacervation with further increase in THF proportion. However, stability of gelatin in co-solvent system at macro level could be due to hydrogen bonding between water and gelatin molecules as shown in Figure 6.3. Further increase in THF has caused coacervation of gelatin molecules. This effect could be due to increase in water-THF interactions, which have decreased the water-gelatin interactions [29]. Each water molecule forms 4 hydrogen bonds with the neighboring water molecules. Addition of THF to water has been reported to decrease the hydrogen bonding among water molecules and increase the water-THF hydrogen bonding. Misaki et al. has studied the intermolecular interactions in water-THF system and demonstrated the decrease in hydrogen bonding of water molecules from ~4 to ~< 0.5 due to with increase in THF mole fraction from 0 to ~0.9 [30]. Intermolecular interactions among water and THF molecules has been demonstrated by Tejraj and co-workers by analyzing the excess molar volume of water-THF system, which remains negative in all water-THF proportions. Large negative values of excess molar volume demonstrate specific intermolecular interactions among water and THF molecules [31].

6.3.2 Structural analysis of GEUPYII in water-THF co-solvent system

From the transmittance studies, it has been observed that gelatin, GEUPYII (0.10) and GEUPYII (0.30) formed a stable clear solution till 43.8% THF beyond which phase separation/ coacervation took place, as shown when the THF proportion was increased to 50% THF. On the other hand, GEUPYII (0.50) could form a stable solution till 50% THF and only undergo phase separation on increasing THF proportion to 52.5% THF. To study the structural transformation in water-THF co-solvent systems, phase separated co-solvent solutions (gelatin, GEUPYII (0.10) and GEUPYII (0.30)) and stable co-solvent solution of GEUPYII (0.50) at 50% THF (0.26261mole fraction) were incubated for 24 hours at 37^{o}C. Incubation at 37^{o}C has changed the color of

solution from milky white to clear due to sedimentation in 50% THF solutions of gelatin, GEUPYII (0.10) and GEUPYII (0.30), whereas no sedimentation took place in GEUPYII (0.50) solution at 50% THF as shown in Figure 6.4. Sedimentation has resulted in formation of a homogeneous film at the bottom of the container and a clear supernatant solution above it.

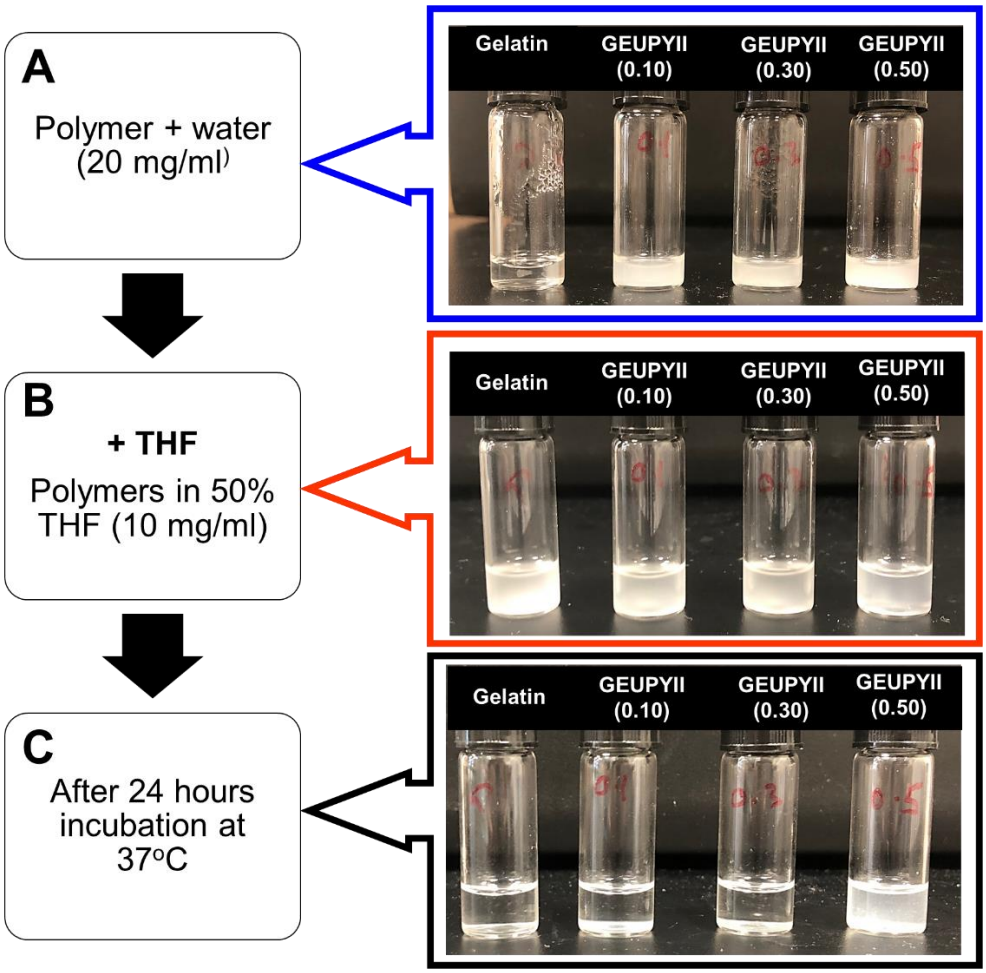

Figure 6.4 – Solutions images of gelatin, GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50) in A) Distilled water (20mg/ml); B) 50% THF (10 mg/ml) C) 50% THF after 24 hours incubation at 37°C (10mg/ml)

Aqueous solutions of gelatin, GEUPY (0.10), GEUPY (0.30) and GEUPY (0.50) were freeze dried for structural analysis by scanning electron microscopy (Picture A, B, C and D of Figure 6.5). And supernatant solutions and films of

gelatin, GEUPYII (0.10) and GEUPYII (0.30) in 50% THF co-solvent were freeze-dried separately to characterize the structural transformations through scanning electron microscopy (SEM), shown in Figure 6.5. In case of GEUPYII (0.50) in 50% THF co-solvent, where no sedimentation took place even after overnight incubation at 37^{o}C, the solution was freeze-dried for structural characterization by SEM (Picture H of Figure 6.5).

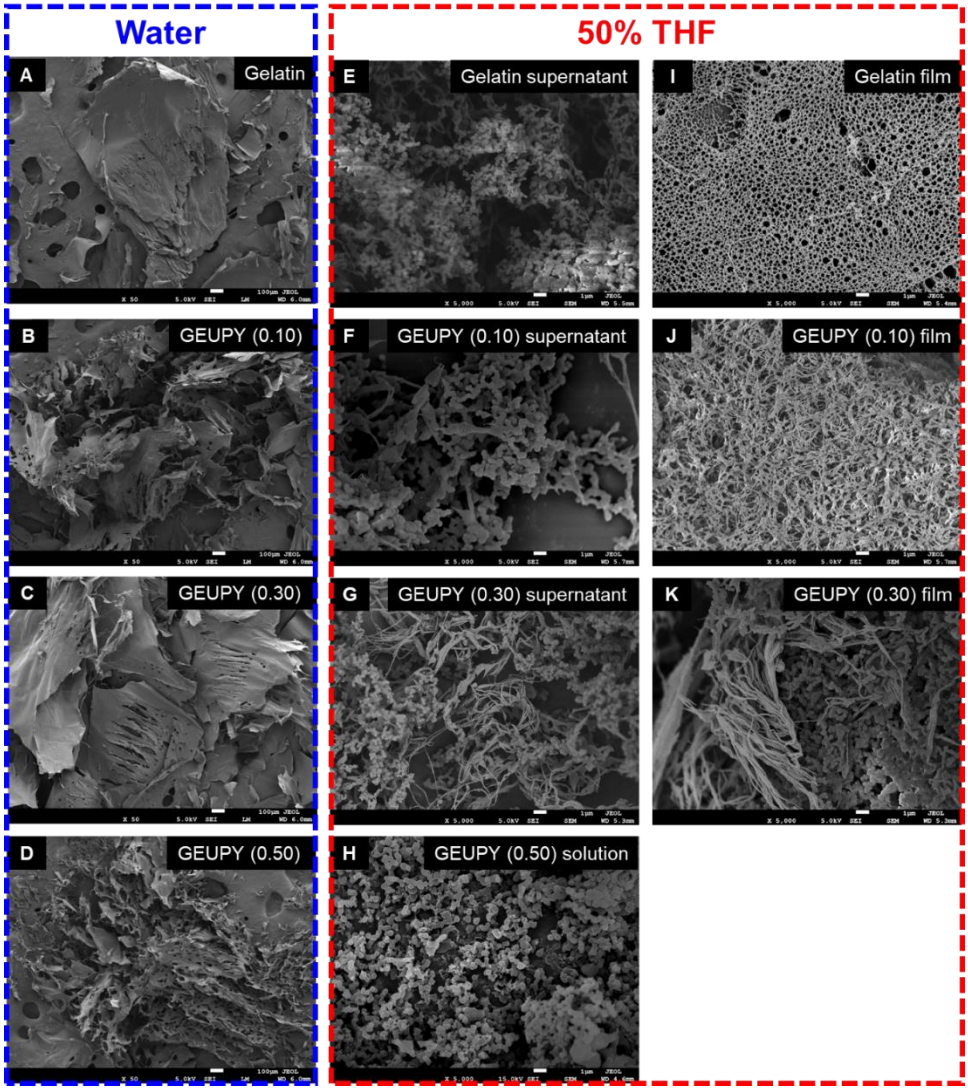

Figure 6.5 - SEM images of freeze-dried powder samples of gelatin (A), GEUPY (0.10) (B), GEUPY (0.30) (C), GEUPY (0.50) (D) in water and gelatin supernatant (E) and film (I); GEUPYII (0.10) supernatant (F) and film (J); GEUPYII (0.30) supernatant (G) and film (K); GEUPYII (0.50) solution (H) in 50% THF (No film formation in GEUPYII (0.50) at 50% THF)

In supernatant solution of gelatin (Figure 6.5(E)) and GEUPY (0.10) (Figure 6.5 (F)), spherical shaped coacervates were present predominantly, other than fused structures. However, fibrous structures in addition to spherically shaped structures were present in solution state of GEUPYII (0.30) (Figure 6.5 (G)).

Similar transition in structures has been observed in Upy substituted poly (ethylene glycol) when pH was adjusted from basic pH to acidic pH due to Upy dimerization [32]. Due to difference in solubility among gelatin, GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50) at 50% THF, size of the micro-structures are incomparable. In case of gelatin, GEUPYII (0.10) and GEUPYII (0.30) at 50% THF, a fraction of biopolymer concentration had undergone phase separation, had sedimented to form a film at the bottom of the container when THF was adjusted from 43.8% THF (x_{THF} (mole fraction of THF) - 0.1478) to 50% THF (x_{THF} - 0.2626). Presence of free THF molecules at 50% THF or 0.26261, x_{THF} could be responsible for the phase separation effect and eventual sedimentation and film formation. Wu et al. has studied the effect of water-THF system on poly (N-isopropyl acrylamide) microgels to study the water-THF systems. Microgels have undergone swelling in pure water and pure THF, whereas undergone shrinking in the range of $0.05 < x_{THF} < 0.15$ due to intermolecular interactions between water and THF. Further increase in x_{THF} resulted in swelling of the microgels. This swelling at higher mole fraction of THF was due to the presence of free THF molecules, which were not hydrogen bonded to water molecules [33]. One THF molecule has been reported to form hydrogen bond with 3 water molecules, due to this the non-hydrogen bonded THF molecules form microdroplets inside the continuous phase of water-THF system [33, 34].

The pattern of coacervates aggregation to form film has been observed to vary with Upy substitution. Gelatin has formed a micro-porous continuous film, whereas GEUPYII (0.10) film was micro-porous but not continuous. This demonstrates that coacervates present in GEUPYII (0.10) were not able to aggregate efficiently to form a continuous film. Micro-porous continuous film formation in case of gelatin at 50% THF could be due to insolubility of gelatin when THF was added and hence gelatin coacervates were precipitated out and fused together to form a film.

Porous non-continuous film formation in case of GEUPYII (0.10) at 50% THF could be due to Upy substitution, which improved the solubility in THF. Due to this, the coacervates were not able to fuse completely in 50% THF. However, GEUPYII (0.30) at 50% THF had similar structures in the film and supernatant. Both supernatant and sedimented film, had fibrous and spherical shaped structures and no fusion of coacervates took place in the sedimented film due to better solvent compatibility. In case of GEUPYII (0.50), structures remained stable in 50% THF solution and did not form a film. Stability of structures could be due to higher Upy substitution, which have better solvent compatibility in comparison to GEUPYII (0.30), since it had higher substitution of Upy.

From the given studies, it can be concluded that the solvent compatibility increases with increase in Upy substitution due to good solubility of Upy in THF. Higher solvent compatibility prevents the fusion of structures in case of GEUPYII (0.10), GEUPYII (0.30) and stability of GEUPYII (0.50) in a water-THF co-solvent environment. Bhosale et al. has reported the structural transformation in water-THF system in AIE active tetraphenylethylene (TPE) derivatives. In the given study, formation of spherical aggregates at 85% THF and formation of fused structures with increase in THF proportion has been reported [35]. Similar spherical aggregates formation has been reported in water THF systems for polymeric systems [36-38]. For reference, aqueous solution of gelatin, GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50) at a concentration of 10 mg/ml were freeze-dried followed by structural characterization by SEM and compared with samples in water-THF co-solvent as shown in Figure 6.5. Presence of THF molecules in water-THF co-solvent had changed the structural association from an aggregated flakes in water to spherical coacervates. In case of GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50), hydrophobic aggregation induced by Upy molecules was segregated by THF molecules present in water-THF co-solvent as shown in SEM studies and *in situ* transmittance studies (Figure 6.2).

6.3.3 Fabrication of stretchable GEUPYII gel via co-solvent system

The most conventional methods used for film fabrication of water-insoluble Upy modified hydrophilic polymers were hot melt pressing and solvent casting. However, these methods are not suitable for polymers which are either heat sensitive of insoluble in organic solvents. Gelatin is a heat sensitive biopolymer and undergoes direct degradation instead of melting upon heating. Due to this, film fabrication cannot be achieved via hot melt pressing. Solvent casting requires the solubility of polymer in inorganic or organic solvent followed by evaporation of solvent to form a film. Upy substituted gelatin derivatives were insoluble in pure organic and inorganic solvents, which makes this method unsuitable for film fabrication.

Upy is a hydrophobic group and introduces hydrophobic interaction in the polymers and its effect has been observed in the transmission studies. For hydrophobically modified polymers, organic solvents have been used to make them soluble. For film fabrication of polymers which has supramolecular interactions, solvent plays an important role. For the formation of supramolecular crosslinks, specific set of conditions need to be fulfilled depending upon the kind of supramolecular interactions. For hydrogel fabrication via supramolecular interactions, solvent should be able to facilitate the formation of supramolecular crosslinks. Formic acid has been known to break Upy crosslinking in modified polymers [39]. Therefore, formic acid was used for the dispersion of Upy modified polymers in solution [21]. Film fabrication of Upy modified gelatin has been carried out by solvent casting in formic acid by Shokrollahi and coworkers. However, the hydrogel obtained by rehydration of dry film has limited stretchability of 11.75% strain at break in fully swollen state. The low stretchability of the hydrogel film could be due to lack of Upy crosslinks, which were unable to form in formic acid. Co-solvent systems have been used by many researchers for the dispersion of hydrophobically modified polymers and block copolymers with hydrophobic

and hydrophilic segments. Dankers et al. have used tetrahydrofuran as co-solvent for the fabrication of the hydrogel. In the given study, PCL-Upy-PEG block copolymer was suspended in co-solvent (1:1 THF:0.9% NaCl aqueous solution), followed by removal of THF to form a hydrogel. Removal of THF cause phase separation and form a viscoelastic hydrogel [40]. Phase separation has been used as a process of film fabrication of Upy modified polymers. Meijer and coworkers have used the pH switching to form a viscoelastic hydrogel via phase separation. PEG was end-modified with Upy and suspended in basic aqueous solution with 0.1M NaOH (pH ~12). Basic solution of NaOH cause tautomerization of Upy and convert it into more soluble form (enol-form). For film fabrication, pH of the polymer solution was adjusted to pH ~3 by addition of 0.1M HCl solution. At pH 3, phase separation took place to from a viscoelastic hydrogel [41].

Here, fabrication of an elastomeric hydrogel from GEUPYII (0.50) using phase separation process was carried out. GEUPYII (0.50) was chosen for film fabrication due to higher substitution of Upy and would form a stable hydrogel in comparison to others. The film fabrication via phase separation was carried out in a stepwise manner to have a better control on phase separation. From the transmission studies, it has been shown that the GEUPYII (0.50) has formed a stable solution till 50% THF. Firstly, aqueous solution of GEUPYII (0.50) was adjusted to 50% THF solution (1:1, water: THF) with continuous stirring, where it turned from milky to clear solution. Further adjustment of THF proportion to 80% to introduce phase separation was carried out by slow addition of THF. Phase separated solution was incubated at 37^{o}C overnight to form an elastomeric gel at the bottom of the container. The solution would turn from milky to clear and an elastomeric film was deposited at the bottom, which was peeled off for further studies (Figure 6.6).

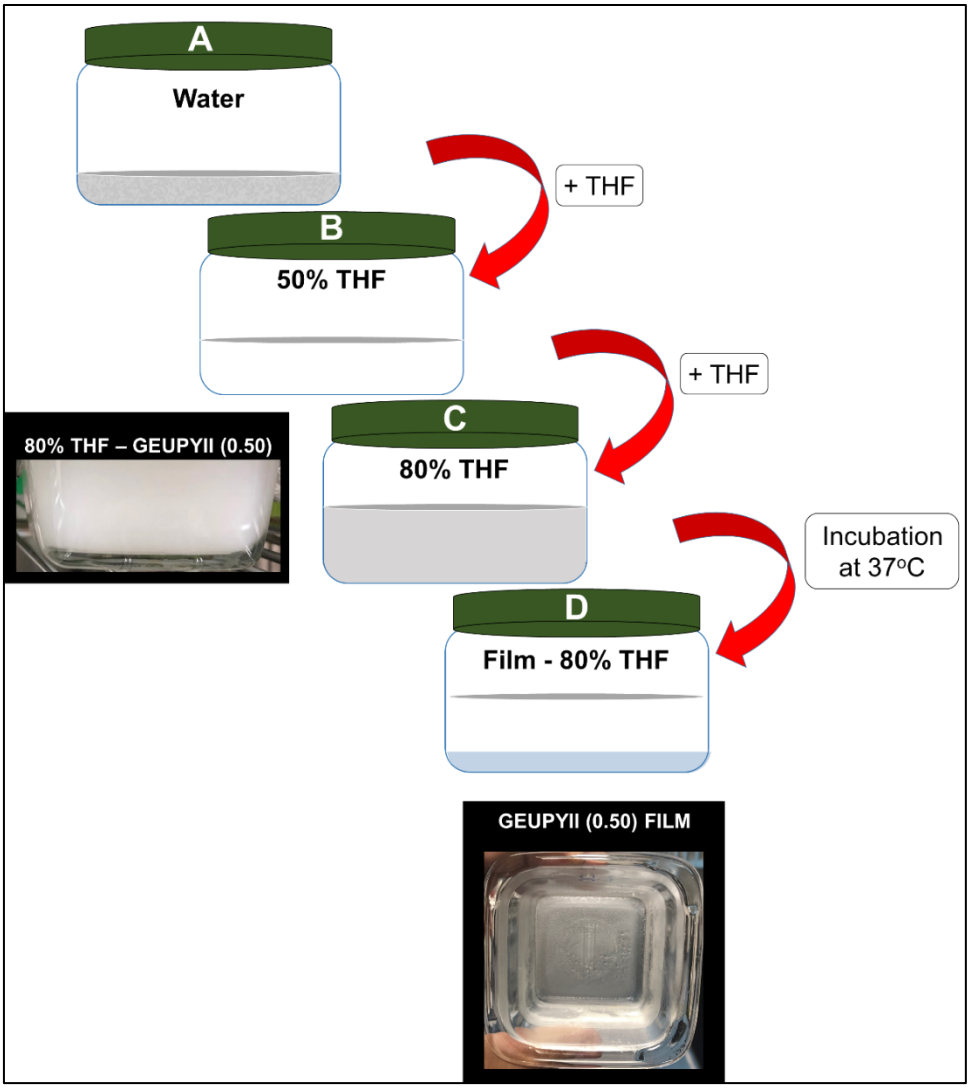

Figure 6.6 Film fabrication method for GEUPYII (0.50) A) Aqueous solution of polymer B) 50% THF solution of polymer C) 80% THF solution of polymer D) Film fabricated after overnight incubation at 37°C.

6.3.4 Structural analysis of gelatin and GEUPYII stretchable gel

Elastomeric film of gelatin and GEUPYII (0.50) was fabricated as described in the section above. The films were observed through optical microscopy and scanning electron microscopy to characterize the structural properties of film. For optical microscopy, the cross-section of both the films were taken and were

observed under optical microscopy at 10X resolution as shown in Figure 6.7. Two different phases were observed in the films A) Continuous polymer rich phase b) Droplet rich phase (which eventually became pores when the solvent in the droplets evaporated during drying). In gelatin film at 80% THF, the droplet phase was present throughout the gel due to phase separation by THF. However, GEUPYII (0.50) gel has less droplets phase in comparison to gelatin film and was present on one edge of the film. Similar droplet phases have been observed by researchers in the polymeric films prepared by phase separation. Dufresne et al. have observed similar droplets in case of gellan gum, alginate and polydimethylacrylamide (PDMA) gels [42].

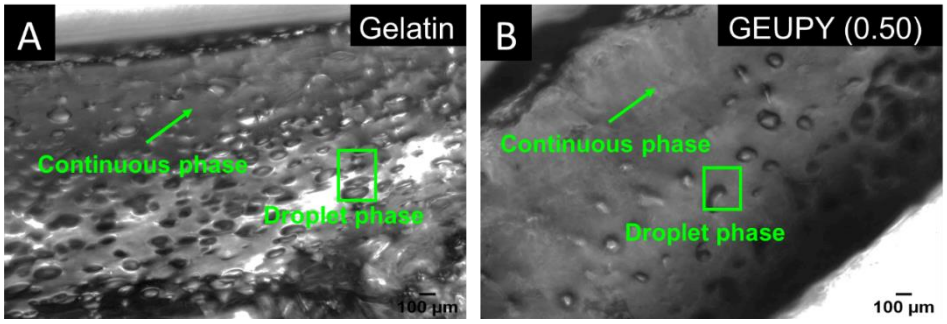

Figure 6.7 Cross-sectional of (A) gelatin and (B) GEUPYII (0.50) fabricated at 80% THF observed by optical microscopy

Freeze-dried films were further characterized for their structural characteristic using SEM. The surface characteristic, pore size and its distribution on the films differs significantly. The surface of GEUPYII (0.50) was solid and does not have any pores as shown in Figure 6.8, whereas pores with size range of 10-65 µm were observed on the surface of gelatin film, with >90% pores size in the range of 10-35 µm.

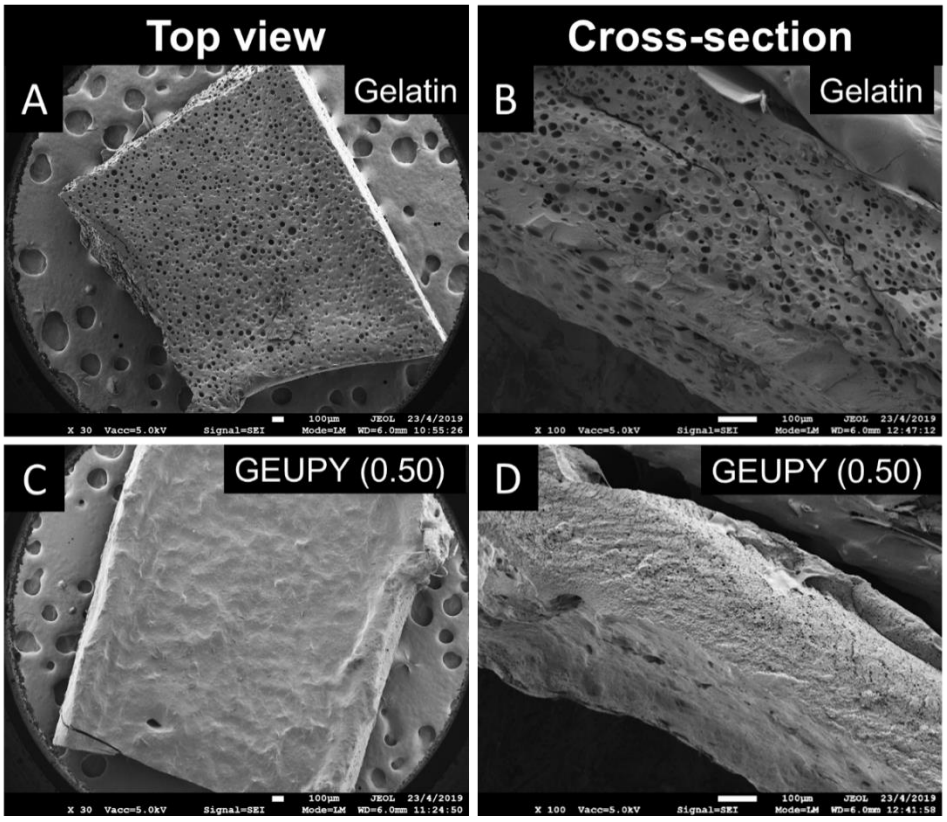

Figure 6.8 SEM images for top view and cross-sectional of freeze-dried film of gelatin film (A- top view, B – cross-section view) and GEUPYII (0.50) film (C- top view, D – cross-section view) fabricated at 80% THF

The cross-section of gelatin 80% THF and GEUPYII (0.50) 80% THF films were further analyzed using ImageJ software for pore size analysis (shown in Figure 6.9). It was observed that the pores were not homogenously distributed throughout the GEUPYII (0.50) 80% THF film cross-section in comparison to gelatin 80% THF film cross-section. The pores were majorly distributed on one edge and were almost absent near to the surface. Also, the pore size of gelatin 80% THF film was ~30 times larger in comparison to the pores of GEUPYII (0.50) 80% THF film. In gelatin 80% THF film cross-section, >80% of the pores have diameter in the range of 10-25 μm and the rest had 30-40 μm, whereas >90% pores of GEUPYII (0.50) 80% THF film cross-section had a diameter in the range of 0.51-1 μm and the rest have 1-2.5 μm. Fewer number

of pores and smaller pore size in GEUPYII (0.50) film could be due to fusion of coacervates in the presence of Upy to form an intact film in comparison to gelatin.

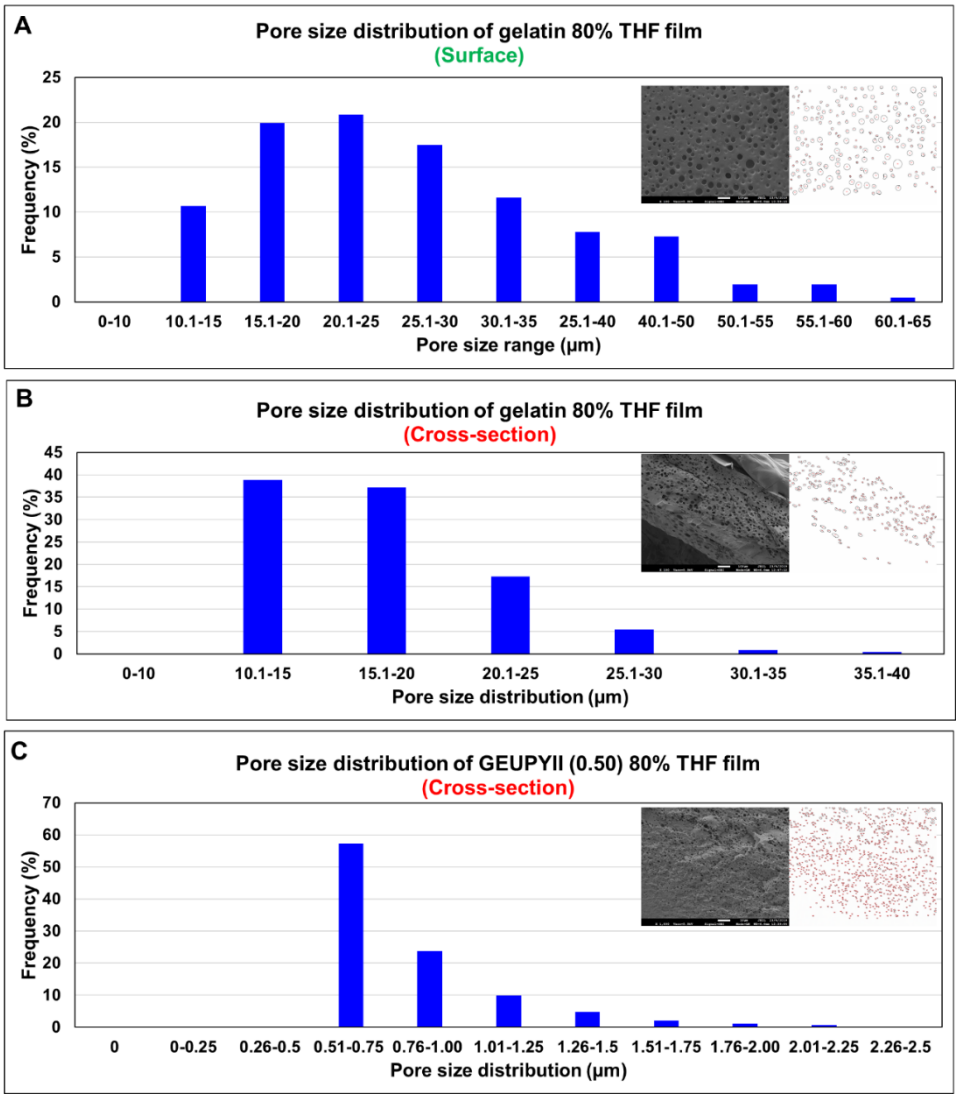

Figure 6.9. Pore size distribution analysis of A) surface pores of gelatin 80% THF film B) Cross-section of gelatin 80% THF film C) Cross-section of GEUPYII (0.50) 80% THF film via ImageJ analysis software

6.3.5 Physical and mechanical properties of elastomeric films

Both gelatin and GEUPYII (0.50) films were initially fabricated by the process mentioned above and further processed to remove water-THF from the film before mechanical property characterization GEUPYII (0.50) 80% THF film has higher stretchability and lower Young's modulus in comparison to gelatin 80% THF film as shown in Table 6.1. Higher stretchability and lower modulus of GEUPYII (0.50) 80% THF films could be due to Upy associations, which involves Upy dimerization and hydrophobic interactions.

Two methods were employed to remove the water-THF co-solvent from the films A) Solvent exchange with distilled water by suspending the film in distilled water for 72 hours; B) Air drying of film in vacuum at 37^{o}C for 48 hours followed by rehydration. In case of gelatin 80% THF film, co-solvent removal by both the methods has swelled and lost its integrity during handling due to lack of crosslinking and were not characterized for their mechanical properties. However, GEUPYII (0.50) 80% THF films remained stable and were characterized for its mechanical properties. Stress vs strain curve for GEUPYII (0.50) film in different states: GEUPYII (0.50) 80% THF, GEUPYII (0.50) SX (72 hours) and GEUPYII (0.50) Rehydrated air-dried film has been shown in Figure 6.10.

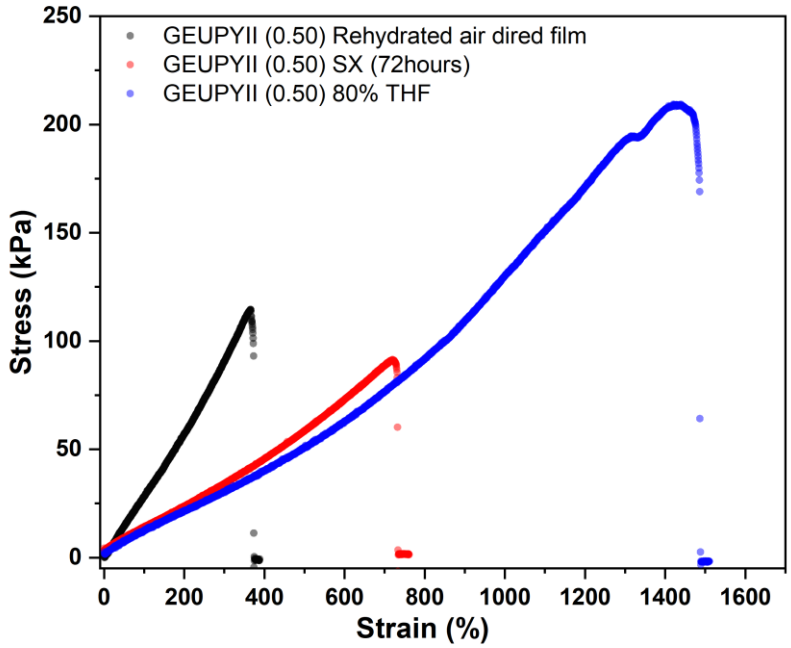

Figure 6.10 Tensile testing of GEUPYII (0.50) film in 80% THF, solvent exchange (72 hours) and rehydrated air-dried films.

Replacement of co-solvent water-THF with water via methods mentioned above has reduced the stretchability and has enhanced the Young's modulus of the GEUPYII (0.50) 80% THF elastomeric films (shown in Table 6.1). The strain at break has reduced from 1405.9 ± 47.9 % for GEUPYII (0.50) 80% THF film to 631.56 ± 109.9% and 318.73 ± 44.4 % for GEUPYII (0.50) SX (72 hours) and GEUPYII (0.50) Rehydrated air-dried film, respectively. However, Young's modulus has increased from 10.13 ± 1.3 kPa for GEUPYII 80% THF film to 12.61 ± 2.6 kPa and 27.35 ± 2.7 kPa for GEUPYII (0.50) SX (72 hours) and GEUPYII (0.50) Rehydrated air-dried film, respectively. Exchange of water-THF co-solvent with water would increase the hydrophobic interactions in the films due to hydrophobic nature of Upy moieties and should be responsible for the increase in Young's modulus and decrease in stretchability.

However, higher stretchability and lower Young's modulus in solvent exchange films in comparison rehydrated air-dried film could be contributed by residual THF molecules present in the film. Water-THF system forms an azeotropic mixture and was difficult to remove it completely by solvent exchange due to hydrogen bonding between water and THF molecules. Air drying of films under vacuum had completely removed the THF from the films in comparison to solvent exchange method. As it has been observed that, the stretchability of GEUPYII (0.50) solvent exchange films has reduced due to partial removal of THF molecules in comparison to GEUPYII (0.50) 80% THF. Complete removal of co-solvent and rehydration of films via air drying method had further decreased the stretchability due to increase in hydrophobic interactions due to hydrophobic Upy molecules substituted over gelatin biopolymer. The stretchability of GEUPYII (0.50) rehydrated air-dried films is still higher than the native gelatin films crosslinked by covalent methods. This increase in stretchability could be due to Upy dimerization took place during the fabrication process and hydrophobic interactions.

Table 6.1 – Strain at break and Young's modulus of GEUPYII (0.50) film in 80% THF, solvent exchange (72 hours) and rehydrated air-dried films

	Gelatin 80% THF film	**GEUPYII (0.50) 80% THF Film**	**GEUPYII (0.50) SX (72 hours)**	**GEUPYII (0.50) Rehydrated air-dried film**
Strain at break (%)	1269 ± 8	1405.9 ± 47.9	631.6 ± 109.9	318.7 ± 44.4
Young's modulus (kPa)	78.71 ± 0.5	10.13 ± 1.3	12.61 ± 2.6	27.35 ± 2.7
Ultimate tensile strength (kPa)	516.7 ± 106.3	207.96 ± 4.8	90.38 ± 60.2	88.57 ± 50.3

6.3.6 Chemical and structural transformation in GEUPYII (0.50) due to co-solvent system during film fabrication

To elucidate the structural and chemical transformation during film fabrication, GEUPYII (0.50) film fabrication was divided into 3 steps and were characterized by SEM microscopy and ATR-FTIR. GEUPYII (0.50) water-THF co-solvent solution with 50% THF was prepared, followed by addition of THF to increase the THF proportion to 52.5%, 60% and 70% THF. After THF adjustment to 52.5%, 60% and 70% THF, phase separated samples were mixed using pipette for 30 seconds and freeze-dried for SEM analysis. In the SEM images shown in Figure 6.11, smaller spherical particles were present at 52.5% THF with size in range of 350-600 nm, whereas at 60% THF larger spherical aggregates with size 3-8 μm were present in addition to smaller particles. Similar large spherical particles with size 3-12 μm were present in 70% THF. The proportion of larger spherical aggregates were higher in 70% THF in comparison to 60% THF. This demonstrates two significant events in the film fabrication. Firstly, the smaller aggregates have fused with one another to form larger spherical aggregates when THF proportion was increased to 60% THF from 50% THF in GEUPYII (0.50). The other one involves increase in proportion of larger spherical aggregates with increase in THF proportion.

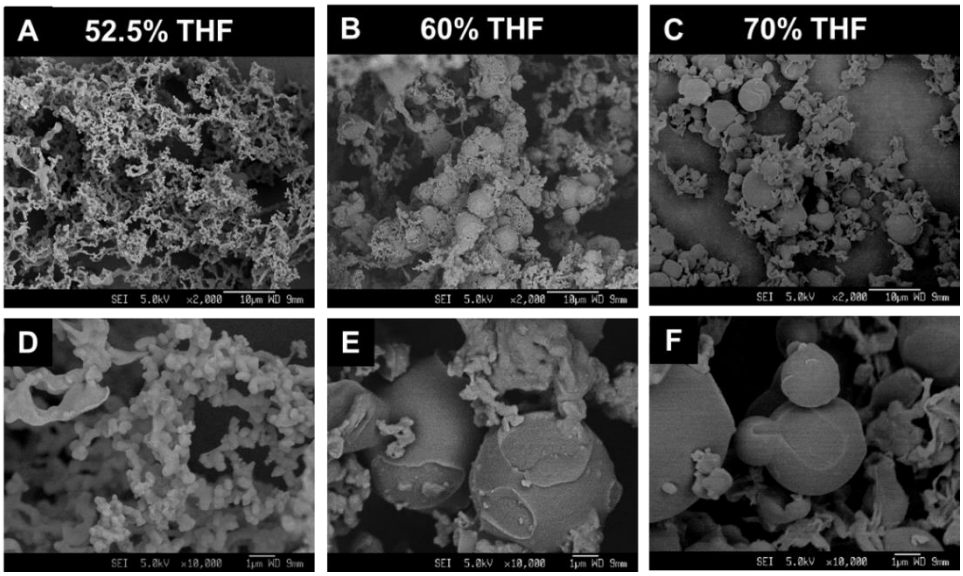

Figure 6.11 SEM of GEUPYII (0.50) at 52.5% (A, D), 60% (B, E) and 70% THF (C, F) after adjusting THF % from 50% THF followed by mixing for 30 seconds and stored at -80^{o}C.

During film fabrication, GEUPYII (0.50) was exposed to water-THF co-solvent system, which caused structural and chemical transformation. To characterize the chemical transformation, freeze-dried GEUPYII (0.50) and GEUPYII (0.50) 80% THF film were analyzed using ATR-FTIR spectroscopy. Transitions due to films fabrication were observed in majorly two regions (I and II) of normalized FTIR spectrum as shown in section A of Figure 6.12 In Figure 6.12, section B and C are enlarged views of region I and II, respectively. In region I, three transitions in the FTIR spectrum were observed, which includes the shift of band at 1439cm^{-1} to 1449.8cm-1 (A), increase in intensity of 1449.8 with respect to 1405.5 cm^{-1} band (B) and decrease in intensity of peak at 1314.2 cm^{-1}(C). FTIR band at 1439 cm^{-1} represents ν(CN) of proline residue in gelatin and is sensitive to backbone conformation [43]. Shifting to higher wavenumber i.e.1449.8 cm^{-1} and increase in its intensity with respect to 1405.5 cm^{-1} band could be due to decrease in intra-chain interaction (due to hydrophobic interactions) to interchain association in the film state. Band at

1314.2 cm^{-1}represents the $\boldsymbol{\delta}$ (CH) (ethoxy group, alkyl chain) of pyrimidino-4-ol tautomers of ureidopyrimidinone. It has been reported as a characteristic band for pyrimidin-4-ol tautomer and absent in 4[1H]-pyrimidinone tautomer of Upy [44]. Decrease in intensity of this could be due to transition from pyrimidin-4-ol to 4[1H]-pyrimidinone. In region II, shift of FTIR bands 2930.5 and 2956 cm^{-1} to 2926.5 and 2952 cm^{-1}, respectively took place due to film fabrication as shown in transition D and E in section C of Figure 6.13. Band at 2930.5 and 2956 cm^{-1} has been reported a characteristic band for C-H stretching of pyrimidin-4-ol, whereas the bands at 2926.5, 2952 cm^{-1} for C-H stretching of 4[1H]-pyrimidinone form [44]. Shift of the band in region II also represents the tautomerization of ureidopyrimidinone. Conclusively, FTIR transitions demonstrates the change in structural organization and tautomerization of Upy from pyrimidin-4-ol to 4[1H]-pyrimidinone (enol to keto) in the process of film fabrication.

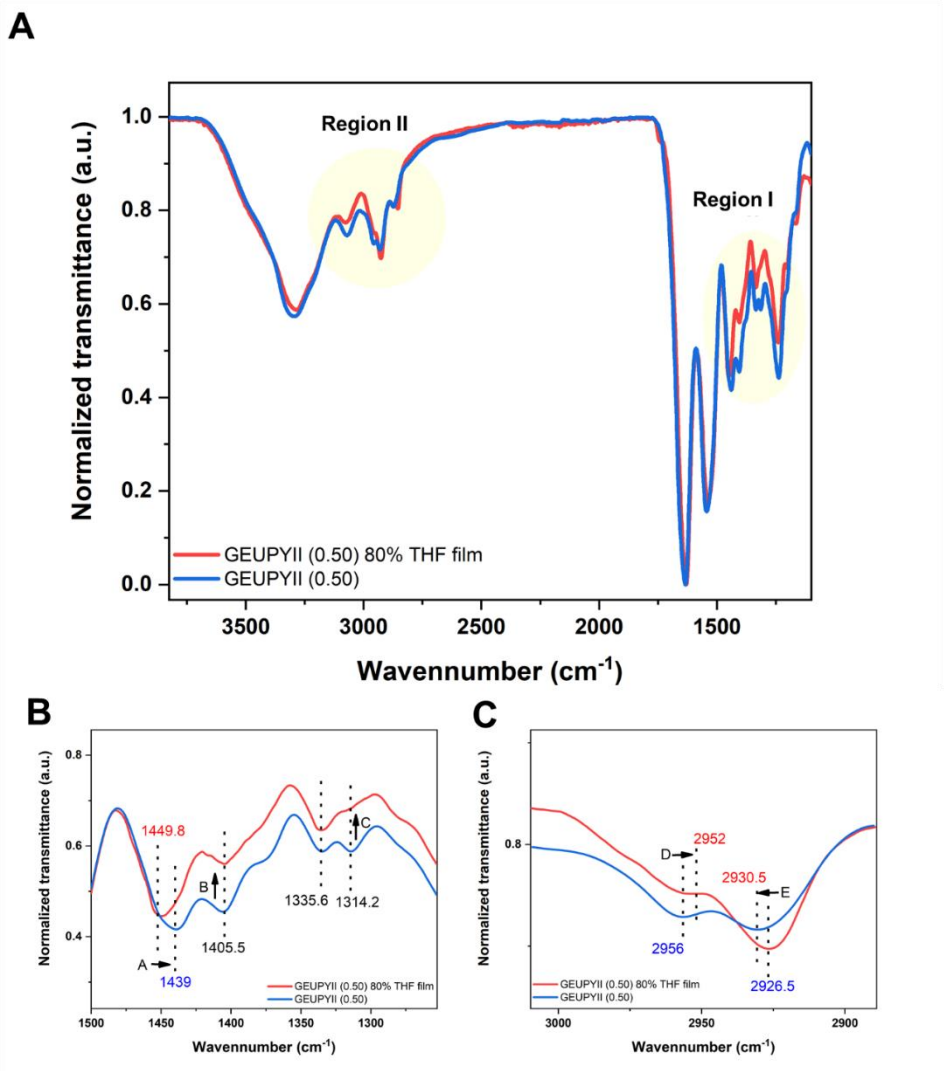

Figure 6.12 ATR-FTIR spectrum of A) GEUPYII (0.50) and GEUPYII (0.50) 80% THF film and transitions at position I and II in FTIR spectrum due to film fabrication are shown in enlarged images shown in section B and C, respectively.

6.3.7 Swelling analysis of GEUPYII stretchable hydrogels

Gelatin and GEUPYII (0.50) film obtained from 80% THF co-solvent was air dried in vacuum at 37°C to analyze the swelling analysis of the film. In both gelatin and GEUPYII (0.50) air dried films the swelling reached to >70% water content in less than 2 minutes as shown in Figure 6.13. Despite the hydrophobic Upy substitution, the swelling of GEUPYII (0.50) film was quite fast and this could be due to pores present in the film. After 3 hours, the water content stabilized in the GEUPYII (0.50) film, whereas for gelatin film, equilibrium was reached after 48 hours at a water content of 91.17± 0.2%. GEUPYII (0.50) film reached saturation at 70.98 ± 1.3%. The lower swelling in the latter is expected due to the more hydrophobic nature of the Upy present.

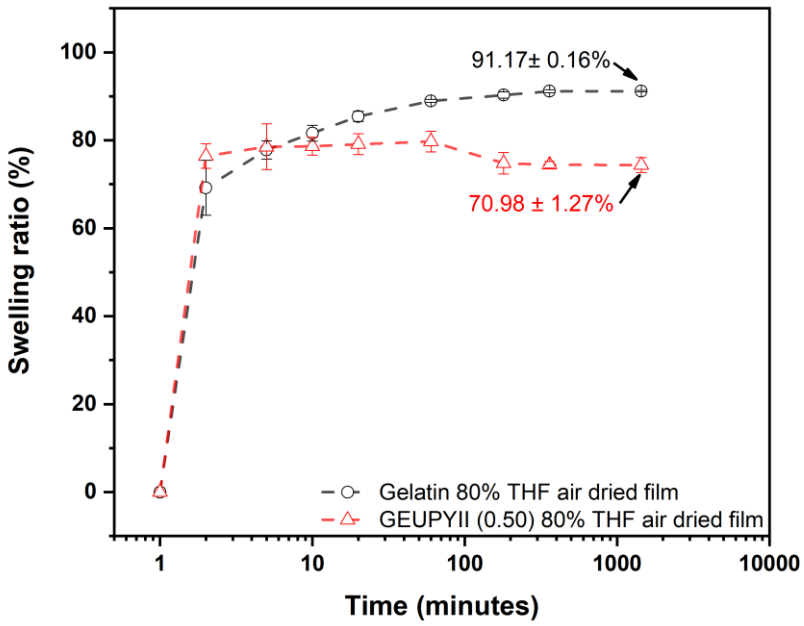

Figure 6.13 Swelling analysis of A) GEUPYII (0.50) and B) gelatin air-dried film fabricated at 80% THF.

6.3.8 Conclusion

In this study, the solubility of gelatin and Upy substituted gelatin derivatives (GEUPYII (0.10), GEUPYII (0.30) and GEUPYII (0.50)) were studied in water-THF co-solvent systems. *In situ* studies of Upy substituted gelatin derivatives in co-solvent solutions demonstrated an increase in transmittance at 600 nm (decrease in turbidity) with increase in THF proportion till a saturation point (saturation point, GEUPYII (0.10), GEUPYII (0.30) - 43.8% THF and GEUPYII (0.50) – 50% THF). The solutions underwent coacervation with further increase in THF proportion. Initial increase in transmittance in Upy substituted gelatin derivatives with increase in THF proportion was due to decrease in hydrophobic aggregation. SEM analysis of freeze-dried samples of Upy substituted gelatin derivatives demonstrated the formation of spherical and fibrous particles/coacervates at 50% THF. THF proportion was further adjusted in GEUPYII (0.50) from 50% THF to 80% THF in co-solvent solutions for elastomeric film fabrication via phase separation. A stable elastomeric film of GEUPYII (0.50) was fabricated at 80% THF with a high strain at break of 1405.92 ± 47.89 %. After solvent removal by air drying method and rehydration, the stretchability decreased to 318.73 ± 44.35 %, whereas Young's modulus has increased from 10.13 ± 1.32 kPa to 27.35 ± 2.69 kPa. Increase in Young's modulus and decrease in strain at break with drying and rehydration could be caused by the micro-phase separation of substituted Upy moieties induced by the water molecules as observed in earlier studies [24]. Elucidation of elastomeric film formation has been studied by microscopic and spectroscopic techniques, which demonstrates the formation of hydrogen bonding between the Upy dimers. Swelling studies of the air-dried film reached >70% water content within 2 minutes. This demonstrates the presence of hydrophilic cavities which has resulted in rapid swelling of the elastomeric films. Conclusively, rehydrated air-dried hydrogel film of GEUPYII (0.50) with 70% water content is a stable hydrogel film fabricated from the co-solvent system.

The stability is better than the gelatin counterpart and is attributed to the presence of supramolecular interactions induced

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Chapter 7

Future recommendations

Chapter 7 demonstrates the future recommendation for the project. Section 7.1 presents the systematic study required to fabricate elastomeric hydrogels with higher Upy substitution and section 7.2 demonstrates the fabrication of a hybrid hydrogel to prevent the drying of GEUPYII hydrogels.

7.1 Systematic study to demonstrate the effect of Upy substitution method on gelatin structure and elastomeric hydrogel properties

Gelatin is a water-soluble hydrophilic polymer due to the presence of charged functional groups (amino and carboxyl) and hydroxyl groups, which introduce electrostatic interactions and hydrogen bonding in aqueous medium. Both charged functional groups and hydroxyl groups plays an important role in the dispersion of gelatin biopolymer in aqueous medium [1, 2]. In the given study the Upy substitution in gelatin biopolymer was carried out in two-steps for its substitution in amino and hydroxyl groups in presence of catalyst as shown in section 4.2 of chapter 4. For synthesis of GEUPUYII, Upy-synthon was synthesized to react with gelatin functional groups. Upy-synthon have an isocyanate group, which requires catalyst to react with hydroxyl group and does not require catalyst for amino groups [3, 4]. This will facilitate site selective substitution of Upy in gelatin biopolymer. The given study will be carried out by reacting Upy synthon with gelatin polymer in presence and absence of catalyst in one step. Absence of catalyst would facilitate the substitution of Upy only at amino groups and presence of catalyst would substitute Upy at hydroxyl groups of gelatin biopolymer. Substitution at amino groups would imbalance the overall charge of gelatin polymer, whereas substitution at hydroxyl groups of gelatin would decrease the hydrogen bonding potential of gelatin biopolymer. Electrostatic interactions are stronger than hydrogen bonding and should have higher influence on dispersibility of gelatin biopolymer in aqueous medium, water-THF co-solvent optimization and elastomeric film fabrication [5, 6]. Upy substituted gelatin polymers with Upy substituted at hydroxyl groups (GEUPYh) should have better dispersibility in aqueous medium in comparison to gelation polymers with Upy substituted at amino groups (GEUPYa).

Upy crosslinking has been increased by researchers by increasing the Upy substitution to enhance the supramolecular crosslinking. However, higher substitution of Upy would lead to loss of water dispersibility in water-soluble hydrophilic polymers, which makes elastomeric film fabrication difficult.

However, GEUPYh aqueous solution should be able to maintain higher transmittance in comparison to GEUPYa even at higher Upy substitution. Due to this gelatin based elastomeric film fabrication with higher Upy substitution could be attempted.

7.2 Biocompatibility of GEUPYII elastomeric hydrogels

GEUPYII elastomeric hydrogels should be investigated for their biocompatibility for tissue engineering applications. Biocompatibility test for GEUPYII elastomeric hydrogels should be carried out by culturing cells over the surface of the hydrogels. Fibroblasts are the mostly widely used cell culture to test the biocompatibility of biomaterials due to monolayer growth. Fibroblast will be cultured over the surface of hydrogels. Growth of cells over the hydrogel surface will be investigated after every 24 hours for 3 days using live-dead assay and confocal microscopy.

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