Protein-Based Hydrogels

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Abstract

Hydrogels have the capability to absorb large amounts of water or biological fluids into their three-dimensional hydrophilic polymer networks. These attractive materials are used to develop food additives, superabsorbents, wound dressing compounds, pharmaceuticals, and biomedical implants and also applied in tissue engineering, regenerative medicines, and controlled-release process. Hydrogels can be obtained from synthetic and/or natural resources. Synthetic hydrogels exhibit high water absorption capacities and proper mechanical strength, although their applications are being limited because of low biocompatibility and biodegradability as well as the toxicity arisen from unreacted monomers remained in the gel structure. Natural hydrogels are often derived from polysaccharides and proteins. Protein-based hydrogels have substantial advantages such as biocompatibility, biodegradability, tunable mechanical properties, molecular binding abilities, and intelligent responses to external stimuli such as pH, ionic strength, and temperature. Therefore, this kind of hydrogels is known as smart biomaterials for controlled release, tissue engineering, regenerative medicine, and other applications. Protein can be converted to hydrogel using physical, chemical, or enzymatic treatments. To improve their mechanical properties, hybrid hydrogels are synthesized by combining natural polymers with synthetic ones. The main approach to obtain hybrid hydrogels is grafting natural polymers with synthetic one and vice versa. This chapter intends to look over protein-based hydrogels. After brief introduction of protein and its structure, the properties of proteins and peptides used to develop hydrogels, as well as their preparation methods are discussed. The potential applications of these polypeptide-based hydrogels in the fields of superabsorbent development, tissue engineering, and controlled release are reported. Characterization methods for protein-based hydrogels are covered in the final section to determine rheological properties, morphology, and thermal stability.

Keywords

Hydrogels · Protein and peptide · Superabsorbent · Controlled release · Tissue engineering · Characterization · Rheological behavior

1 Introduction

Hydrogel-based technologies have been flourished in numerous fields upon the introduction of hydrogels in 1954. Inherent 3D structure of hydrogels absorbing large amount of water is composed of hydrophilic polymers. The main features of hydrogels are their insolubility and stability in aqueous solutions due to the presence of cross-linked polymer chains. Moreover, their water holding capacity varies from 1 to 1000 g/g absorbent. The mechanical properties of hydrogels can be tailor-made using chemical and physical cross-linking. Based on the cross-link nature, hydrogels are categorized as physical or chemical ones. Physical hydrogels are reversible and

stabilized by ionic and hydrogen bonds as well as hydrophobic forces. Changes in environmental conditions including pH and temperature, and addition of solutes may disrupt such hydrogels. In contrast, chemical cross-linking methods arrange covalent bonds in the body of hydrogels and make them more stable [1–8].

The first generation of hydrogels was mainly based on hydroxyalkyl methacrylate and its derivatives creating swelling capacities up to 40–50%. This kind of hydrogels was used to develop contact lenses. Nowadays, hydrogels have a wide range of applications. These attractive materials have many applications in food additives, superabsorbents, wound dressing, pharmaceuticals, biomedical implants, tissue engineering, regenerative medicines, diagnostics, controlled drug delivery, wastewater treatment, and biosensor. In addition to agricultural usage, superabsorbency of hydrogels makes them applicable for developing personal care and hygienic products such as disposable diapers, sanitary napkins, towels, sponges, and surgical pads. Hydrogels can be affected by the changes in pH, temperature, the concentration of metabolite, and osmotic pressure. In addition, specific molecules such as glucose or antigens can stimulate hydrogels. Such hydrogels can be used in biosensors and controlled-release delivery systems for drugs and chemicals [5, 9–14].

Synthetic polymers such as poly(acrylamide), polyacrylate, poly(hydroxyalkyl methacrylates), and poly(methacrylamide) and its derivatives, poly(N-vinyl-2-pyrolidone) and poly(vinyl alcohol), are currently exploited to prepare hydrogels. Although this kind of hydrogels exhibits high water absorption capacities and excellent mechanical strength, their applications are being limited because of the toxicity arisen from unreacted monomers presented in the gel structure. Moreover, low biodegradability and biocompatibility of the synthetic hydrogels may cause environmental problems [15, 16]. To overcome these challenges, natural-driven hydrogels can be proper alternatives.

In contrast to synthetic ones, natural hydrogels are biodegradable and compatible with living cell activity and often remain safe byproducts. However, natural hydrogels have inherent disadvantages such as poor mechanical strength and immune inflammatory responses. Additionally, the natural hydrogel compositions vary batch to batch; and biomaterials with animal origin, especially proteins, may transfer viruses or other pathogens to the site of application, which is so crucial in biomedical engineering applications. Polysaccharides-based and proteins-based hydrogels are the most popular examples of the natural hydrogels [17].

Proteins have inherent advantages over polysaccharides for hydrogel developments. Proteins contain several functional groups including amino, carboxyl, hydroxyl, sulfhydryl, and phenolic groups, which can act as reactive sites for chemical modifications and cross-linking. Proteins are nontoxic, biocompatible, and biodegradable. Proteins and peptides are often incorporated into hydrogels to design biomimetic materials for tissue engineering and drug delivery, since polypeptides are natural constituent of the extracellular matrix. However, some sorts of proteinous hydrogels, e.g., thermally induced ones, cannot re-swell to their original volume after drying due to the increment of protein–protein interactions via hydrogen bonding and electrostatic and hydrophobic interactions occurring as a result of dehydration. Therefore, it is crucial to enhance the swelling and water-absorbing

properties of proteinous gels, which can be obtained by appropriate chemical modifications of the constituents [6, 9, 15]. This chapter aims to review protein-based hydrogels, which is initiated by brief description of protein and its structure. Afterward, the properties of proteins and peptides as hydrogel constituents, and the corresponding hydrogel preparation methods are considered. Superabsorbent developed by protein-based hydrogels as well as their applications in tissue engineering and controlled release are reported. Rheological properties, morphology, and thermal stability of proteinous hydrogels are highlighted in the final section.

2 Protein Structure and Properties

To form a peptide bond, two amino acid molecules covalently link together through the condensation reaction between an amino group of one molecule with an carboxyl group of another one. The reaction results in the elimination of a water molecule and the formation of a dipeptide. A short sequence of amino acids is called peptide while protein, also known as polypeptide, refers to longer chains of amino acids. Amino acids in the structure of peptides or proteins are called residues to be distinguished with the free form. Proteins may contain 20 different types of amino acids which are joined to the protein backbone by peptide bonds. Amino acids are classified as polar, nonpolar, aromatic, anionic, and cationic. The amino and carboxylic groups in amino acids have weak basic and acidic characters, respectively. These groups are found in the ionized forms, arranging zwitterionic form of amino acids [9, 18, 19].

Proteins are essential macromolecules found in all living systems from bacteria to vertebrates and higher mammals. Over 50 percent of the cell dry weight can be proteins. Through covalent or non-covalent bonds, proteins can be attached to other biomolecules such as carbohydrates, lipids, phosphate groups, nucleic acids, flavins, and metal ions. Proteins are synthesized based on the information conserved in the genetic material of cells such as DNA. Replication, transcription, and translation are the main processes for synthesizing protein in living cells [18, 20, 21].

The proteins exhibit four types of structure including the primary structure (amino acid sequence), the secondary structure (conformation), the tertiary structure (overall folding of the polypeptide chain), and the quaternary structure (specific association of multiple polypeptide chains). The amino acids are connected through peptide bonds to form a linear sequence of amino acids, known as the primary structure. The secondary structure is obtained after the protein biosynthesis, where the supramolecular interactions such as hydrogen bonds and ionic, van der Waals, and π interactions arrange the local conformation of proteins. The secondary structure of proteins comprises mainly α-helices, β-sheets, and β-turns. The nature, bioactivity, and hydrophobicity of the proteins depend on amino acid compositions which is responsible for the folding of secondary structures into the three-dimensional tertiary structure. Nonlocal interactions, salt bridges, hydrogen bonds, and disulfide bonds cause stabilization of tertiary structure. In short, the secondary structure shows the spatial arrangement of neighboring amino acids in the peptide while the tertiary one explains the overall spatial arrangement of the protein. Non-covalent bonding into

the tertiary structures creates more complex assemblies known as quaternary structures. Several factors such as temperature, pH, organic solvents, and disulfide bond highly influence non-covalent bonds and disulfide bonds found throughout the secondary, tertiary, and quaternary structures. Consequently, these factors affect protein structures and functionalities. Based on functionality, protein can be categorized in several classes such as structural proteins and enzymes. Structural polypeptides act as the main constituent of feathers, hair, muscles, and silk to support integrity in the tissues and organs [9, 18, 22, 23]. Nowadays, proteins are used to develop many innovative and smart hydrogels. In the following sections, several protein and peptide-based hydrogels are explained.

3 Protein-Based Hydrogels

Proteins have been used attractively as raw materials to develop several biomaterials, for example, hydrogels, films, and composites. Hydrogels are produced in different forms such as cubic, hollow tube, rod, sheet, and film, considering their applications. Fibrous protein-based hydrogels offer similar structural, mechanical, and chemical properties to the native extracellular matrix, and these biomaterials can be processed simply under mild conditions to act in harmony with living cell. On the other side, proteolytic enzymes can degrade proteinous hydrogels. Such properties make them fascinating to be used in the field of tissue engineering [16, 24, 25]. To develop this kind of biomaterials, protein can be obtained from natural origin or prepared through biosynthetic routes. Peptides are also used to develop peptide-based hydrogels. The applied peptides are often chemically synthesized.

Protein can be converted to hydrogel using physical (cooling, heating, high pressure), chemical (acidification and addition of salt), or enzymatic treatments. Unfolding of the native protein structure and aggregation into a gel network is the most governing mechanism in protein gelation process. The formed network is able to hold water within its structure. The gel network can be stabilized through non-covalent cross-links such as hydrophobic and electrostatic interactions, and hydrogen bonds. Alternatively, a covalent cross-linking can stabilize the gel network [26, 27].

To modify mechanical strength and thermal stability of protein-based hydrogels, chemical cross-linking is carried out using many chemicals. Concentration of cross-linker influences hydrogel mechanical and release properties, as well as degradation rate. All proteins have the potential to be cross-linked, especially via amine and carboxylic acid functional groups. Amines are found at the N-terminus although lysine and arginine residues have additional amine groups. On the other hand, carboxylic acids are located at the C-terminus in addition to glutamic and aspartic acid residues. Other functional groups such as hydroxyl and sulfhydryl ones can be involved in cross-linking; but these groups are not abundant in comparison with amine and carboxylic acid groups. Protein-based hydrogels are often cross-linked by aldehydes and carbodiimides coupling amine-carboxylic acid. Both cross-linkers are toxic to living cells, so these compounds are not useful for cell encapsulation. To overcome this challenge, polyethylene glycol (PEG) with aldehyde or activated

ester groups is used for cross-linking. Remaining large amount of PEG in the hydrogel alters the properties of the gel, for instance it may increase the mechanical strength and swelling ratio. Genipin, a small molecule originated from gardenia fruits, is able to react with primary amines. It has been used to naturally cross-link several hydrogels based on collagen, gelatin, fibrin, and silk. However, carbodiimide and genipin are safer than aldehyde for cross-linking [28–30].

Whereas natural and synthetic hydrogels exhibited many limitations, development of hybrid hydrogels by combining natural polymers with synthetic polymers was emerged to customize chemical, physical, and biological properties of such hydrogels. The main approach to obtain hybrid hydrogels is grafting natural polymers with synthetic ones and vice versa. The grafted polymers can be cross-linked to form hydrogel network. Cross-linking a mixture of natural and synthetic polymer is another way to produce hybrid hydrogels. This kind of hydrogels can integrate the advantage of both synthetic and natural polymers to adjust physical properties, cross-linking ability, bio-adhesiveness ability, and biodegradability. In other words, chemical and physical properties of hydrogels can be adapted for particular applications with the conserving of biocompatibility, biodegradability, and functionality of the natural moiety. Nowadays, many proteins have been using to obtain hydrogels. In the next sections, the properties of collagen and gelatin, soy protein, silk, zein, keratin, casein, albumin, elastin, resilin, lysozyme, and peptides as well as their corresponding hydrogels are briefly described [17, 31–33].

3.1 Collagen and Gelatin–Based Hydrogels

Collagen as a natural polymer is found in extracellular matrices. Animal skin, bones, and articular tissues contain collagen and constitute about 30% of the total protein content in mammals. Collagens are mostly found in the fibrous form, and play important role in mechanical functions throughout the body. In connective and bone tissues, collagens offer biochemical properties needed for proper functioning. Furthermore, collagens reversibly interact with cellular mediators such as cytokines and growth factors. A triple helix including a repeat of amino acid sequence of -Glycine-Xaa-Yaa is the structural element of collagen, in which Xaa and Yaa often stand for proline and hydroxyproline, respectively. Collagen can be exploited in its native fibrillar form. The denatured collagens are suitable to fabricate several hydrogel forms such as sheets, tablets, pellets, and sponges [34–38].

Different animals such as cow and pig are used to extract collagen. The composition, solubility, transparency, mechanical strength, thermal stability, and rheological properties of collagen depend on the extraction sources. Gelatin is prepared by breaking triple-helix structure of collagen into single-strand molecules. Gelatin is thermo-responsive, and therefore it can display a sol-gel transition feature. Wherever dropping temperature occurs, the transition from solution to gel takes place. This change can be reversed by heating the mixture to physiological temperature. Gelatin-based hydrogels are nonimmunogenic, biodegradable, and biocompatible. Thus, this kind of hydrogels is known as an interesting candidate for biomedical applications.

The low thermal and mechanical stabilities of gelatin-based hydrogels can be improved by chemical modifications such as cross-linking. Gelatin-based materials have already been using in tissue engineering applications [39–43].

Collagen-based hydrogels can be prepared using physical treatments. Heating and increasing pH cause the aggregation of collagen through covalent bond to reform the fibrils and to create the hydrogel network. However, the derived hydrogels lack mechanical strength since collagen in native tissues has both intramolecular and intermolecular covalent bonds through lysine and hydroxylysine residues. Additional cross-linking is considered the main solution to overcome this challenge. Glutaraldehyde, genipin, carbodiimide, and gamma radiation have been used to cross-link collagen hydrogels. To customize the properties of collagen-based hydrogels, several materials were employed for the synthesizing of hybrid and composite collagen hydrogels. For examples, poly(N-isopropylacrylamide) and 2-methacryloyloxyethyl phosphorylcholine were used to produce such hybrid hydrogels. On the other hand, cellulose, hydroxyapatite, and silver nanoparticles have been applied to prepare composite collagen hydrogels [44–47].

3.2 Soy–Based Hydrogels

Soybean is a well-known food source, containing 40% protein. Glycinin and β-conglycinin are the main constituents of soy protein. This globular protein has proper gelling and foaming abilities, needed for preparing porous hydrogels. Open and interconnected pores, improved surface area, and high swelling capacity are features of the porous hydrogels. Soy-based hydrogels can be prepared using physical, chemical, or enzymatic cross-linking. The hydrogels prepared by thermal or cold treatments have weak mechanical structure and degrade fast. In contrast, chemical or enzymatic cross-linking can improve the structural strength. Chemical cross-linkers such as glutaraldehyde and genipin have been used to prepare soy-based hydrogels. Furthermore, zein and collagen in combination with soy protein isolates have been used to prepare the hybrid hydrogels. Soy hydrogels have been used for developing superabsorbents and biomaterials [44, 48–57].

3.3 Silk–Based Hydrogels

Silk proteins are extracted from the cocoons of silkworms, although the silk glands also contain these proteins. The composition and structure of silk proteins depend on silkworm species. Two classes of protein, fibroin and sericin, mainly constitute silk originated from silkworms. *Bombyx mori* fibroin has the repetitive sequence of a hexapeptide. This hydrophobic hexapeptide creates fibroin crystallinity and stability. Sericin is highly hydrophilic and consists of 18 amino acid residues. Sericin composition, solubility, and structural organization make it suitable for cross-linking, copolymerizing, and combining with the other polymers. Association of the crystalline regions with peptide domains increases the strength and elasticity of silk protein fibers;

therefore fibroin fibers can form films, sponges, gels, and tubes. Silk-based biomaterials can be applied in biomedical engineering because of their biocompatibility with living cells. Silk fibroin contains several hydrophobic amino acid residues like glycine, serine, and alanine. Consequently, it can form hydrogels using physical cross-linking methods such as temperature change, decreasing pH, and phase separation. The applied method and fibroin concentration influence gelation rate. For example, gel formation may occur faster with increasing the temperature or fibroin concentration. Hybrid silk-based hydrogels have been prepared using many natural and synthetic polymers such as alginate, chitosan, gelatin, collagen, and polyacrylamide. Genetically engineered silk proteins in combination with elastin-like polymers have been used to prepare silk-elastin-like polymers. Strength, immunogenicity, solubility, and degradation rate of silk-elastin hydrogels can be tailored with manipulation of amino acid compositions of both proteins. Repetitive silk peptides provide mechanical strength to the copolymer, while elastin peptides are responsible for flexibility, solubility, and cross-link density of the related hydrogels [58–69].

3.4 Zein-Based Hydrogels

Zein is a hydrophobic protein, found in the endosperm of corn kernel along with other proteins like glutelin, globulins, and albumins. Since zein is insoluble in water, solvents such as alcohol, anionic detergents, or urea are used to dissolve it. Zein can be self-assembled to several forms such as chains, layers, or foams via different processing methods. Zein is generally recognized as a safe biomaterial with the promising properties like biocompatibility, biodegradability, and non-toxicity. Because of low nutritional value, zein has great potential to be used as a precursor for the development of biomaterials. Particularly, zein-based hydrogels can be employed for manufacturing superabsorbents and drug delivery. For such applications, the zein should be solubilized in water, which can be performed through chemical modifications. For example, citric acid and acetic anhydride have been used to chemically modify zein, and the process is followed by electrospinning. The modified protein was soluble in neutral phosphate buffer solution. After cross-linking with sodium hexametaphosphate, the protein formed a hydrogel with stimuli-responsive behavior toward pH and ionic strength. Hybrid zein-based hydrogels can be prepared by combining with other polymers. For example, pectin–zein hydrogels have been developed by mixing a pectin solution with a zein solution in ethanol. Additionally, a solution of zein and soy protein isolates dispersed in soybean oil has been reported for the synthesizing of a hybrid zein hydrogel [70–80].

3.5 Keratin–Based Hydrogels

Keratin, a fibrous protein, is found in animal hairs, nails, wool, horns, and feathers, which can be divided into two groups of soft and hard keratin. Epidermal keratin is known as soft one, while hairs, nails, horns, and feathers are the sources of hard keratin.

α-Helix and β-sheet constitute keratin conformations. Keratin contains 14 types of amino acids with great amount of cysteine in its composition. For example, feather keratin and wool keratin have about 7% and 17% cysteine, respectively. As a result, many cross-links exist in the protein structure due to the formation of inter- and intramolecular disulfide bonds. Therefore, keratin is insoluble in water and has relatively high mechanical strength. Comparing with collagen, keratin is not degraded simply in vivo, since there is no keratinase in animal body to degrade keratin. Therefore, keratin-based hydrogels can be natural alternative to develop long-lasting biomaterials and superabsorbents [81–87]. Keratin is mainly extracted from wool, human hairs, and chicken feathers; all of them contain keratin more than 90% by weight. To synthesize the hydrogel, keratin solution should be cross-linked by a chemical agent or by promoting the formation of disulfide bonds. For example, H_2O_2 solution was added to keratin solution to stimulate the formation of these bonds. The resulting mixture was incubated over night to form gels. In another study, sodium bisulfite/potassium persulfate as the initiators were added to hydrolyzed feather keratin solution. Then, the protein solution was mixed with the mixture of methylenebis (acrylamide) and acrylic acid to prepare a keratin-poly acrylic acid hydrogel [82, 88, 89].

3.6 Casein-Based Hydrogels

Casein, the major proteinous constituent of milk, contains about 94% protein which is in combination with colloidal calcium phosphate. Cow milk has mainly four amphiphilic casein phosphoproteins. Casein hydrogels can be exploited in drug delivery systems because of interesting properties of casein such as high hydrophilicity, biocompatibility, edibility, and chemically modifiable structure. Disadvantages of casein may include its possible immunogenicity and allergenicity. Casein-based hydrogels can be prepared via chemical cross-linking by genipin in an aqueous environment. The mechanical strength of the cross-linked casein hydrogels can be customized by the amount of genipin. Enzymatic cross-linking is another approach to prepare casein hydrogels. Transglutaminase was used under mild conditions for gelation of milk proteins. The prepared hydrogel was suitable for biomedical applications. Hybrid casein hydrogels were also prepared through graft copolymerization using methyl methacrylate monomers. Furthermore, a pH-dependent hybrid hydrogel was synthesized using a mixture of acrylamide and methylenebis(acrylamide) with casein [90, 91].

3.7 Albumin–Based Hydrogels

Serum albumin is known as the most abundant globular protein in blood. It contains 580 amino acid residues. The protein consists of 54% α-helix and 40% β-sheet in its structure. Serum albumin acts as a transport protein for numerous molecules. Bovine serum albumin, with structural homology to human serum albumin, is an abundant and low cost globular protein with medical importance. Ovalbumin and

β-lactoglobulin are the other important albumins. Albumins have been used to prepare hydrogels or other biomaterials [36, 92–95].

3.8 Elastin–Based Hydrogels

Elastin, an extracellular matrix protein, gives tissues the properties of elasticity and strength. It is composed of about 800 amino acid residues, containing highly hydrophobic and cross-linking domains. Therefore, soluble forms of elastin including tropoelastin, α-elastin, and elastin-like polypeptides (ELPs) are frequently used to develop hydrogels. In addition, the elastin should be purified for biomedical applications. ELPs are referred to the polymers, the building blocks of which are synthesized in accordance with human elastin sequences. ELPs contain a pentapeptide repeat, Valine-Proline-Glycine-X-Glycine, in which X can be any natural amino acids, except proline. ELPs are thermally responsive, and can hydrophobically self-associate with increasing the temperature. ELPs do not stimulate immune system and are biocompatible. Thus these polypeptides have a great potential for application in drug delivery or tissue engineering [4, 36, 96, 97].

Chemical, enzymatic, and physical cross-linking approaches have been tested to prepare elastin-based hydrogels. For example, lysine-containing ELPs were rapidly cross-linked to form hydrogel by tris(hydroxymethyl)phosphine propionic acid. Clearly, the number of lysine residues in the peptide influences the mechanical properties of the hydrogel [96, 98].

3.9 Resilin–Based Hydrogels

Resilin, the most stretchable elastomeric protein, is found in insect cuticles. Resilin originated from dragonfly tendons can be stretched up to three times of its original length before breaking. The resilin-related gene in *Drosophila* encodes 620 amino acids. The expressed protein contains tyrosine and glycine residues which are responsible for cross-linking and flexibility of the polypeptide chains. Resilin-like hydrogels were generated by genetic engineering of resilin-encoding genes to be used as a support for drugs and enzymes. The resilin-based hydrogels were prepared by an enzymatic cross-linking method via horseradish peroxidase. In order to improve photochemical cross-linking ability, modification of the resilin by replacing tyrosine with phenylalanine was also reported. A hybrid hydrogel was produced by cross-linking of cysteine residues on resilin and vinyl sulfone-terminated PEG. Such hydrogels have been applied for biomedical engineering [25, 99–102].

3.10 Lysozyme–Based Hydrogels

Lysozyme, containing 129 amino acids, is a small globular protein with α-helix and β-sheet in its secondary structure. This protein is highly soluble in water and found in

egg white, animal tissues, tears, and milk. Egg white lysozyme cannot be self-assembled at pH 7. To overcome this challenge, dithiothreitol, a reducing agent is used to disrupt the disulfide bridges in lysozyme structure. This agent unfolds lysozyme and consequently induces the protein self-assembly. To form hydrogel at neutral pH, the solution of lysozyme and dithiothreitol heats to 85 °C and then cools slowly to room temperature [36, 90, 103, 104].

3.11 Peptide–Based Hydrogels

Peptides as well as proteins are often used in the development of hydrogels. The incorporation of peptides into biomaterials can be carried out with a high level of chemical specificity in contrast to proteins which often incorporate into biomaterials via nonspecific amine−carboxylic acid couplings. Peptide-based hydrogels show advantages for biomedical application such as binding ability to cells, growth factors and surfaces, and biodegradability [105].

Poly(aspartic acid) has several advantages such as hydrophobicity, biocompatibility, and biodegradability. It displays extensive potential applications in water treatment, cleaning products, and sanitary, agricultural, and biomedical fields. Poly (aspartic acid) hydrogels are synthesized by mild alkaline hydrolysis of the polysuccinimide gels. Poly(aspartic acid) hydrogels show a pH-dependent water absorption capacity [106, 107]. The gelation of poly(aspartic acid) can also be performed using co-polymerization, cross-linking, and radiation polymerization. For example, poly(aspartic acid) gels were prepared by chemical cross-linking polysuccinimide with 1,4-diaminobutane through a solid–liquid phase separation technique at cryogenic condition. In another study, cross-linking of this peptide using gamma radiation led to a high swelling ratio. A hybrid poly(aspartic acid) hydrogel was prepared by exploiting acrylic acid as a monomer, persulfates as an initiator, and methylenebisacrylamide and tetra-methylenebisacrylamides as cross-linkers. Furthermore, another hybrid hydrogel was prepared with combination of hyaluronic acid and polyaspartic acid. Other homo-poly(amino acids)s such as poly(lysine) and poly (glutamic acid) have also been applied to prepare hydrogels [108, 109].

4 Applications of Protein–Based Hydrogels

Protein and peptide-based hydrogels have many different applications in superabsorbent development, tissue engineering, and controlled release, which are described below.

4.1 Superabsorbent Hydrogels Based on Proteins

Superabsorbent hydrogels displaying exceptional water absorption ability are generally developed using ionic monomers which are lightly cross-linked. Water is

found in a hydrogel network in different forms. It can be free or bulk water present in the hydrogel exterior region being easily removable under natural conditions, water physically trapped into polymeric network called interstitial water, chemically bound water hydrating hydrogel functional moieties being directly attached to polymeric chains, and semi-bound water representing the form between the extremely bound and free water [14, 17]. Superabsorbent hydrogels are interestingly used as water-saving materials for agricultural uses especially for the revival of desert environment. Such hydrogels have the ability to reduce water consumption, to enhance fertilizer availability in soil and to help plant growth. Furthermore, superabsorbent hydrogels are widely applied as hygienic materials for instance disposable diapers and lady napkins to absorb blood and urine [110, 111]. Super-swelling hydrogels can be synthetic or natural-based. Natural superabsorbent hydrogels are mainly developed using polysaccharides or proteins. Cellulose, starch, and chitosan are examples of polysaccharides for preparing superabsorbent hydrogels [112–115]. Several proteins and peptides have been reported for developing superabsorbent hydrogels. Such hydrogels can be obtained by cross-linking of proteins after grafting with hydrophilic groups.

Collagen has been employed to prepare several hybrid superabsorbent hydrogels. As an efficient way to increase hydrophilicity, protein backbones were grafted with vinylic monomers and followed by cross-linking of the copolymer. For example, collagen and gelatin were used to develop such hydrogels, and a maximum water absorbency of 920 g/g was obtained using the hydrolyzed collagen-based hydrogel [116–118]. A collagen/kaolin-based hydrogel composite was synthesized through graft copolymerization of acrylic acid onto the protein. In this experiment, methylene bisacrylamide as a cross-linker and ammonium persulfate as an initiator were used. The maximum water absorbency was about 674 g/g [119]. Graft copolymerization of 2-acrylamido-2-methylpropane sulfonic acid and acrylamide into a hydrolyzed collagen in the presence of sodium montmorillonite was reported to prepare a superabsorbent nanocomposite. The optimum water absorbency was 681 g/g [120]. A hybrid hydrogel showing high swelling capacity was developed based on a mixture of kappa-carrageenan and gelatin via graft copolymerization of acrylamide. The hybrid superabsorbent hydrogel displayed a maximum water absorbency of 3310 g/g [121]. In another research, a superabsorbent hydrogel was developed by grafting a mixture of salep/gelatin with acrylamide. At the optimum condition, a maximum water absorbency of 762 g/g was obtained [122]. Recently, γ-irradiation in the absence of oxygen was employed to develop a hybrid superabsorbent hydrogel based on collagen–polyvinylpyrrolidone. A swelling capacity in the range of 2000% was obtained for this hydrogels at best [123].

Superabsorbent hydrogels containing –OH, $–NH_2$, $–CONH_2$, –COOH, and $–SO_3$ groups can adsorb many molecular and ionic species such as cationic dyes and metals [10]. Some of these groups are found in protein-based hydrogels, particularly in hybrid hydrogels. A hybrid superabsorbent hydrogel based on acrylic acid and the hydrolyzed collagen was synthesized through graft copolymerization. The hydrogel was able to uptake bivalent metal ions such as copper, cobalt, nickel, and zinc. It showed the highest affinity to copper ion, with a sorption capacity of 1.39 mmol/g,

while the maximum water absorbency was 500 g/g [118]. A poly(acrylic acid) was grafted into gelatin backbone to fabricate a granular hydrogel. The hydrogel was able to adsorb malachite green with the maximum adsorption capacity of 1370 mg/g [124]. A hydrogel based on gelatin, carboxylic acid functionalized multi-walled carbon nanotube and iron oxide magnetic nanoparticles was developed to adsorb anionic Direct Red 80 and cationic methylene blue dyes from aqueous solutions. The adsorbent showed a capacity of 465 and 380 mg/g for methylene blue and direct red, respectively [125].

Soy protein has been considered as a proper material to synthesize natural-based superabsorbent since it is easily available and cheap with highly hydrophilic character. Additionally, soy protein in combination with a plasticizer can be processed to form different shapes [126–128]. Manufacturing of cross-linked microcapsules as a novel catamenial absorbent using soybean protein through a solvent evaporation technique was reported. The hydrogel was able to absorb plasma up to 2000% [129]. In another study, soy protein isolate and potassium acrylate were used to synthesize a hybrid superabsorbent hydrogel. Primary amino groups in alkali-treated soy protein were functionalized using methacrylic anhydride by free radical copolymerization in a one-pot process. The functionalized soy protein had cross-linking ability [130]. A biodegradable poly-anionic hydrogel was derived through chemical modification of lysyl residues of soy protein isolate with ethylenediaminetetraacetic dianhydride as an acylating agent, followed by cross-linking with glutaraldehyde. The hydrogel was able to absorb water up to 300 g/g dry gel [15, 131]. Such a modification created a large number of carboxylate anions into the biopolymer [132]. As a result, numerous binding sites with hydrophilic character appeared. Acylating soy protein can also be performed with succinic anhydride. In order to obtain a superabsorbent hydrogel, succinic anhydride functionalized protein was mixed thoroughly with glycerol, and the process followed by molding the hydrogel. Water uptake capacity of the acylated hydrogel was higher than that of untreated protein [133].

A silk-based superabsorbent hydrogel was synthesized with graft copolymerization of acrylic acid and acrylamide onto the sericin chain in silk. Potassium persulfate and sodium sulfite as redox initiators and methylenebisacrylamide as a cross-linker were used. The hybrid hydrogel showed a maximum swelling capacity of 2150 g/g in water [62]. In the similar experiment, a synthesized silk sericin-g-poly(acrylic acid/attapulgite) hydrogel had a water uptake capacity of 1236 g/g [134]. Cryogels are macro-porous hydrogels formed below the freezing point of its solvent. Zein was used to prepare cryogels by graft copolymerization of acrylic acid onto the protein backbones, where acrylamide as a cross-linker and sodium bisulfite/potassium persulfate as initiators were employed. The absorption capacity of the cryogel for water and diesel fuel was about 120 and 50 g/g, respectively [79]. In another study, a sodium alginate/zein hydrogel was synthesized on a polypropylene fiber. The hydrogel assembly was able to extract polar compounds such as ethinyl estradiol, progesterone, and estriol [135]. Hydrolyzed keratin grafted by acrylic acid monomers was used to prepare a hybrid hydrogel in the presence of methylenebis (acrylamide) as a cross-linker and sodium bisulfite/potassium persulfate as initiators. The maximum swelling capacity of the hydrogel in distilled water was about 500 g/g dry hydrogel [82].

Several hydrogels based on renneted caseinate, sodium caseinate cross-linked with transglutaminase, and casein micelles cross-linked with transglutaminase were prepared, and their water holding capacities were investigated [136]. A physically cross-linked hydrogel composed of polyvinyl alcohol and casein was prepared via freezing–thawing treatment of aqueous solutions. Swelling ratio of the cryogel in water was about 14 g/g dry hydrogel [137]. A copolymer hydrogel, the blocks of which are based on elastin-like protein, was synthesized. The maximum cadmium binding capacity of hydrogel was 1.3 nmol Cd/nmol protein. The hydrogel showed reversibility in metal binding experiments [138]. A protein-based hydrogel system containing a network of engineered uranyl binding proteins and elastin-like polypeptides which was assembled through thiol-maleimide click chemistry was developed to enrich uranyl from natural seawater with great efficiency and selectivity [139].

The other proteins originated from fish, canola, and cottonseed were also used to develop protein-based superabsorbent hydrogels. Fish processing plants generate considerable amount of proteinous waste, which can be used for preparing superabsorbent hydrogels. A hydrogel based on fish protein was synthesized by introduction of hydrophilic groups into fish protein through modifying the protein with ethylenediaminetetraacetic dianhydride followed by cross-linking with glutaraldehyde. The hydrogel showed a water uptake capacity of about 200 g/g dry gel. Treatment of hydrogel with absolute ethanol increased the uptake capacity to 425 g/g [140]. In the similar experiment, the water uptake of a modified fish protein-based hydrogel was 540 g/g dry gel [141]. The synthesis of a canola protein-based hydrogel was reported through solution-based graft copolymerization of the canola protein backbones with acrylic acid monomers. In this reaction, methylenebis (acrylymide) and sodium bisulfite/potassium persulfate were used as a cross-linker and initiators, respectively. The maximum swelling capacity was 448 g/g dry hydrogel in distilled water [16]. A protein-based superabsorbent hydrogel was prepared through graft copolymerization of hydrolyzed cottonseed protein with acrylic acid. In the copolymerization reaction, methylene bisacrylamide as a cross-linking agent and potassium persulfate/sodium sulfite as the initiators were used. The hydrogel showed the maximum water absorbency of 890 g/g [112]. A cottonseed protein-poly(acrylic acid) copolymer hydrogel composite was synthesized to adsorb copper and lead ions from aqueous solution. In single component sorption experiments, the hydrogel composite showed sorption capacities about 3 mmol/g for each ions [142].

Peptides have also been used to synthesize superabsorbent hydrogels. For example, amino acid homopolymers such as poly(aspartic acid)s, poly(lysine)s and poly (glutamic acid)s are reported for developing such hydrogels. Ethylene glycol diglycidylether and polyethylene glycol diglycidylether were employed as cross-likers to synthesize poly(aspartic acid) hydrogels with super-swelling behavior [143, 144]. To improve swelling ability, several chemicals such as starch, carrageenan, and polyacrylamide were incorporated into poly(aspartic acid) hydrogels [145]. The modification of poly(succinimide)s with dimethylformamide resulted in allyl-containing poly(aspartic acid)s. The modified poly(aspartic acid)s were converted to hydrogels by chemical cross-linking, using ammonium persulfate/potassium

peroxodisulfate as radical initiators. Such hydrogels with low allyl group content offered water absorption capacity up to 424 g/g [146]. In another research, L-aspartic acid was used to modify starch, and the experiments were followed by hybrid hydrogel preparation. The resulting hydrogel was a superabsorbent with pH and temperature-responsive swelling behavior. The equilibrium water content of the hydrogel was more than 5000% [147]. To prepare lysine-based hydrogel, γ-irradiation on poly(lysine) aqueous solution was performed to lightly cross-link the polymer. The hydrogel showed a swelling capacity of 160 g/g. A hydrogel was also prepared by the γ-irradiation of poly(glutamic acid). The specific water content of this hydrogel was about 3500 g/g dry hydrogel. The results indicated that the hydrogel swelling capacity decreased when the irradiation dose increased [148]. Propyl esterification of the carboxyl groups of poly(glutamate) was employed to synthesize thermosensitive biopolymers. The poly(propyl glutamate) was formed hydrogel after cross-linking with hexamethylene diisocyanate. The maximum swelling ratio was 71% based on gel dry weight [149]. Recently, a superabsorbent hydrogel based on poly(glutamic acid) was synthesized through solution polymerization. Cross-linking was performed using ethylene glycol diglycidyl ether. Maximum swelling capacity in NaCl solution was 21 g/g [150].

4.2 Protein-Based Hydrogels in Biomedical Fields

Finding materials to mimic the native environment with the appropriate chemical and mechanical properties is a crucial challenge for tissue engineering and drug delivery. Hydrogels are able to mimic soft tissue since their properties can be modified through designating the nature of functional groups and controlling monomer and cross-linker concentrations before initiation of the gelation process [6, 58]. Hydrogels particularly proteinous one can be sensitive to the changes in environment factors such as pH, temperature, or metabolite concentrations and release their load in situ. It makes them useful as controlled-release delivery systems for bioactive agents [14]. On the other hand, proteins are one of the main constituents in the extracellular matrix. Consequently, polypeptides can be precisely incorporated into hydrogels, either by stable covalent bonds or through transient non-covalent interactions [6]. Protein-based hydrogels are known as promising biomaterials for many applications in the biomedical fields.

4.2.1 Tissue Engineering

Hydrogels are attractive for tissue engineering applications since they exhibit particular features. As a matrix, the advantages include their aqueous environment, the ability to transport biochemicals and to entrap living cells, and ease of modification and application [4, 151]. Collagen, the structural protein in the extracellular matrix, is known as one of the most applicable biopolymers for tissue engineering. In addition to inherent properties of hydrogels, collagen inserts excellent sites for attachment of adherent cells to the hydrogels, and displays in vivo biodegradability [33].

Collagen-based hydrogels were used to develop scaffolds for creating synthetic tissues such as blood vessel [152], spinal cord [153], cartilage [154], heart valves [155], and skin [156]. This kind of hydrogel is also applied for delivering bioactive factor to induce the chondrogenesis of stem cells [157, 158]. It is reported a collagen/hydroxyapatite hydrogel used as a scaffold for differentiation of stem cells to form bone in vitro and in vivo [159, 160]. The collagen sponges in combination with mesenchymal stem cells have been successfully applied to repair tendon defects in rabbits [161]. Collagen-based hydrogels are attractive in wound dressing applications because of their ability to temporarily repair damaged tissues [162]. A gelatin/alginate hydrogel cross-linked by carbodiimide, containing montmorillonite and kaolin, was used as bio-adhesives support. It may be applicable in hemorrhagic environment [163]. However, the applications of such hydrogels are limited because of the rapid degradation in vivo and low mechanical strength [164], which can be overcame by the appropriate cross-linking and hybridizing [165–168]. For example, an elastin-like polypeptide–collagen hybrid hydrogel was used as a polymeric matrix for encapsulation of mouse pre-osteoblastic cells. The results revealed high cell viabilities, cell attachment, and proliferation [168]. A reticulate matrix of hyaluronic acid hydrogel coated with collagen was employed as reconstructive tumor models for cancer researches [169].

Gelatin, derived from collagen, with nonimmunogenic, biodegradable, and biocompatible properties, are known as a candidate for tissue engineering. However, poor mechanical properties of gelatin enforce extensive cross-linking especially using tyrosinase and transglutaminase. Gelatin hydrogels were synthesized through enzymatic cross-linking with transglutaminase. The hydrogel characteristics such as cell adhesion, proliferation, and differentiation using an adipose tissue derived from stromal cells were evaluated in vitro. The results confirmed an improvement in the characteristics of the hydrogel [36, 170–172]. Gelatin-based hydrogels in combination with graphene oxide and carbon nanotubes have been used for cell encapsulation [173, 174]. A nanofibrous scaffold based on gelatin hydrogel containing bone-like apatite has been synthesized. The scaffolds showed an improvement in differentiation of the osteoblastic cells [175]. In another study, gelatin/alginate hybrid hydrogels containing hydroxyapatite were developed and used as a scaffold for bone and chondral tissue engineering [176]. A dextran/gelatin hybrid hydrogel containing a growth factor was used as a vehicle and controlled-release system for the differentiation of mesenchymal stem cells to nucleus pulposus [177]. Gelatin hydrogels prepared by cross-linking via lysine diisocyanate ethyl ester were developed. The results proved the biocompatibility of the hydrogels during in vivo study with mice [178]. Microspheres developed from styrene/gelatin hybrid hydrogel were loaded by growth factors and insulin. The beads were successfully applied for adipose tissue repair in rats [179].

A non-woven soy fiber was incorporated in a poly(vinyl alcohol) hydrogel through freezing–thawing cycles to manufacture a hydrogel scaffold. The results proved an increase in mechanical robustness of the hydrogel and cytocompatibility of the scaffolds for cellular attachment [180]. A fibroin hydrogel, inoculated with chondrocytes isolated from white rabbits, was synthesized and used as a scaffold to

evaluate its performance on connective tissue regeneration. The cells growth was observed in the fibroin hydrogel [181]. In another study, silk fibroin hydrogels were applied to treat bone defects in rabbits. The hydrogels showed biodegradability with no inflammatory effects. Bone healing and stimulated cell proliferation were also observed [182]. A hybrid hydrogel based on fibroin/sodium alginate has been developed to control mineralization of hydroxyapatite crystals in bone repair [183]. The use of a silk fibroin hydrogel as a scaffold to study adhesion and proliferation of human and animal cell lines was also reported [184–186]. Using a keratin hydrogel as a cell or growth factor delivery vehicle was described to regenerate functional muscle in the volumetric muscle loss injury in the rat [187].

Porous hydrogel scaffolds based on casein/bovine serum albumin were developed through the calcium-induced cold gelation technique. The scaffolds with cell adhesion and proliferation abilities were suitable for tissue engineering [94]. Bovine serum albumin was applied to develop a protein-based hydrogel. In vitro study proved the appropriateness of the hydrogel for tissue engineering since the prepared gels did not affect the viability of two cell models [93].

Development of highly cytocompatible and injectable elastin-based hydrogels with alterable gelation characteristics was reported. A thermoresponsive succinimide ester-functionalized copolymer was grafted with elastin through covalent bond formation. The prepared hydrogel was injectable and showed proper structural stability, mechanical properties, and live cell proliferation ability. The unique properties of the elastin-based hydrogel made it favorable for tissue engineering applications [188]. In another study, a photocross-linked elastin-like polypeptide gel was synthesized. The biocompatibility of the hydrogels was proved in vivo with subcutaneous implantation of hydrogels in rats. The hydrogel acted as a hemostatic material in vivo [189]. Hydrogels derived from elastin-like polypeptides were successfully used for treatment of articular cartilage damage, in vitro [190].

Other proteins such as resilin and lysozyme have also been exploited for hydrogel preparation. Preparing a resilin-based hydrogel using a modular protein was reported. The hydrogel was obtained by cross-linking the protein with tris (hydroxymethyl)phosphine and applied in cultivation of human mesenchymal stem cells. The results proved suitable cell spreading on the hydrogel [101]. In another research, a resilin-like hydrogel was successfully used to encapsulate human mesenchymal stem cells and aortic adventitial fibroblast cells [100, 191]. The lysozyme gels were used as scaffolds for culturing fibroblast cells. The results indicated proper cell proliferation and spreading [103, 104]. Additionally, application of soy-based hydrogels in orthopedics has been reported [49].

Protein domains, known as either distinct functional or structural units in a protein, are widespread in nature. These domains fold into three-dimensional structures and have been used as scaffolds for tissue engineering. For example, physical cross-linking of a triblock protein using leucine zipper coiled-coils to create cell binding scaffolds has been reported. Human cells were used to evaluate the hydrogel system. The results confirmed that the system was an attractive tool for developing cell-specific surface with tailored functional features [192, 193]. A synthetic hydrogel was obtained using a short building-block derived from the collagen sequence

through sol–gel polymerization. The hydrogel had the potential as a biomimetic scaffold for the stem cell proliferation [11].

Another peptide-functionalized hydrogel was synthesized through copolymerization of poly(ethylene glycol) diacrylates and methacrylated peptides. The hydrogel showed substantial affinity to human cells supporting its applicability in the field of tissue engineering [194]. A biocompatible and self-assembling peptide hydrogel was successfully developed as a cell carrier and scaffold for culturing nucleus pulposus cells [195].

4.2.2 Controlled Release

The delivery of the active agents such as drugs and DNA in a predetermined course of release is known as controlled release, which can occur in either sustained or targeted forms. In the field of drug release, providing adequate amounts of chemicals at a targeted area for known period is crucial [58]. Such a delivery is possible with the protein-based hydrogels.

Collagen has been applied as a delivery system. Collagen/fibrin microbeads were constructed and used as a delivery system for dental pulp stem cells in dental applications [196]. Mechanically robust soy protein hydrogels were synthesized with no cross-linkers. Drug releasing capability of the hydrogels was suitable using fluorescein. The hydrogel biocompatibility was proved through viability and growth of mouse fibroblast cells. The soy hydrogels were a promising biomaterial for drug delivery applications [50]. Silk-based hydrogels have been applied for sustained release of bevacizumab, a therapeutic for certain cancers [197]. Hybrid hydrogels based on fibroin/polyacrylamide have been evaluated for drug release. The results indicated that these hydrogels were applicable for sustained release of trypan-blue [198]. Since successful gene delivery is the key in gene therapy, several protein-based hydrogels were used as DNA delivery systems. For example, silk-elastin like polymers have been used as a DNA delivery system for cancer gene therapy [199, 200].

The use of a zein-based gelling system carrying pingyangmycin was reported. The drug was successfully released in vitro and in vivo [201]. In another study, a zein-based hydrogel loaded with doxorubicin was used for interstitial chemotherapy [80]. Fabrication of keratin hydrogels with tunable rates of erosion was reported for controlled release of a growth factor. The study proved the properness of the hydrogel for control drug and cell delivery [202].

Casein and polyacrylamide have been used to develop hybrid hydrogels to evaluate the releasing of bromocresol green. The results confirmed that casein had the potential to be used in the human body without any toxic effect [203]. A hydrogel with the potential for controlled release of hydrophilic drugs was prepared by cross-linking casein using oxidized hyaluronic acid containing aldehyde groups. In vitro cytotoxicity studies confirmed biocompatibility of the hydrogel [204].

A pH-sensitive hydrogel based on bovine serum albumin using hydroxyethyl methacrylate and acrylic acid monomers was prepared by gamma irradiation. The hydrogel showed high equilibrium swelling ratio up to 1550%. It was successfully

used for the release of flutamide [205]. In another study, a pH and redox sensitive albumin hydrogel was successfully used for in vitro tetracycline delivery under non-reducing and reducing conditions [92].

To enhance mechanical properties, a peptide-based hydrogel was synthesized through chemical cross-linking with genipin. The hydrogel loaded by naproxen showed its drug release ability in aqueous medium [206]. In another study, a pH-sensitive peptide hydrogel was developed as a biocompatible glucose-responsive insulin delivery system. The hydrogel was loaded with glucose oxidase, catalase and insulin. The peptide could self-assemble into a hydrogel form under physiological conditions. The system showed the ability to regulate the blood glucose levels in mice models [207].

5 Characterization Techniques of Protein-Based Hydrogels

Generally, there is a wide range of tests to detect and characterize the protein-based hydrogel, depending on their structure (such as the presence of nano-materials as filler or in the structure as crosslinker, etc.) and application types (behavioral change due to the environmental factors such as temperature, pH, stress or strain action, etc.). In this chapter, in order to identify the characteristics of the protein-based hydrogels, the characterization tests are divided into three parts: rheology, morphology, and thermal stability of protein-based hydrogel, as shown in Fig. 1.

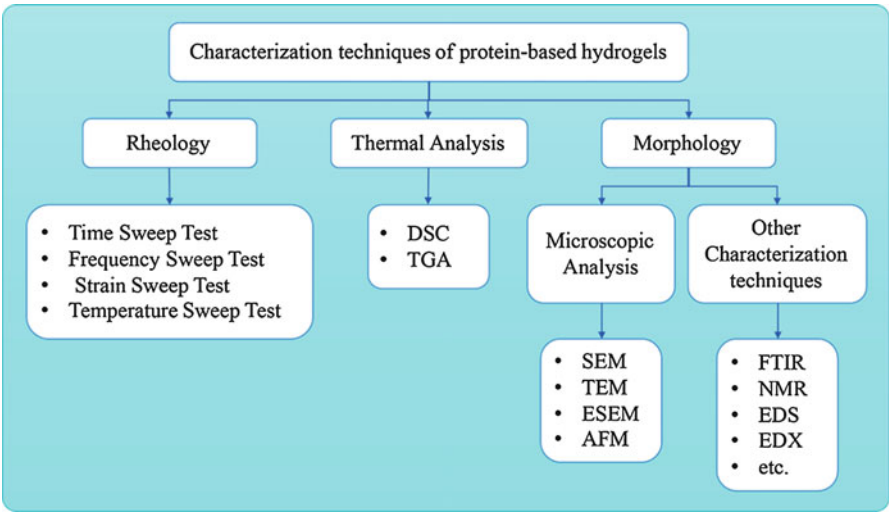

Fig. 1 A classification chart of characterization tests of protein-based hydrogels

5.1 Rheological Characterization of Protein-Based Hydrogels

When it comes to viscoelastic and low properties of hydrogels based on peptides, proteins, and polymers, rheological characterization can be the first significant candidate tool to cover more information. Moreover, rheology produces a straight view of different types and magnitudes of parameters effects (pH, salt, cations/anions, temperature, and enzyme) on the behavior of these hydrogels in various mechanical and biological environments. This part of this chapter proves the critical value of rheological characterization in order to configure the hydrogel characteristics such as gelation time, gel strength, viscoelastic character, and yield-strain behavior. Common rheological studies conducted on hydrogel materials include measurement of storage modulus (G', qualitatively the material stiffness), loss modulus (G'', qualitatively the material liquid-like low properties), and loss factor (tan (δ), the ratio of liquid-like behavior to solid-like behavior), all measured as functions of time, oscillatory frequency, and oscillatory strain. These studies on hydrogels can explain a complete tailor-made properties view on gelation kinetics, linear viscoelastic regions, and relaxation timescales.

The constant stress test is not suitable to analyze the rheological behavior of viscoelastic fluids because the results are not repeated in same conditions of subsequent tests. This unrecoverable results causes from two main reasons [208]. Firstly, the Wiesenberger effect, leading the fluid to rise from the rotary shaft (without discharging the contents of the container). Secondly, fluid resistance toward the flow, leading the constant stress, damages the natural network of the gel. Based on these features, oscillatory measurement instruments are most suggested to observe the gelation process with the aim of minimizing its effects on the severity of the reaction and without any influence on the three-dimensional gel structure. A common method to determine the viscoelastic property of material is the stress measurement along with sinusoidal alternate shear strain and a typical characterization method of viscoelastic gels is oscillation rheology. It is a technique to investigate the presence of reversible interactions and, by varying the frequency of deformation, the possibility of viscoelastic properties at various time scales. In an oscillation rheology measurement, sinusoidal shear deformation is applied and the resulting stress is measured as a function of time. The main parameters of the experiment are the amplitude of oscillation (γ_0) and the frequency of oscillation (ω, i.e., angular frequency) [209]. The correlation between strain (γ) and stress (τ) can be expressed as follows:

$$\gamma(t) = \gamma_0 \sin(\omega t) \tag{1}$$

$$\tau(t) = \tau_0 \sin(\omega t + \delta) \tag{2}$$

where δ is the phase difference between the two waves which is $\delta = 0$ for elastic solids and $\delta = 90^\circ$ for Newtonian fluids. Figure 2 shows a model of strain wave. If the material is viscoelastic, the phase difference will be in between of these two degrees. For proper implementation of test, the stress wave is usually divided into two waves with the equal frequency. One wave illustrates the elastic component (τ'),

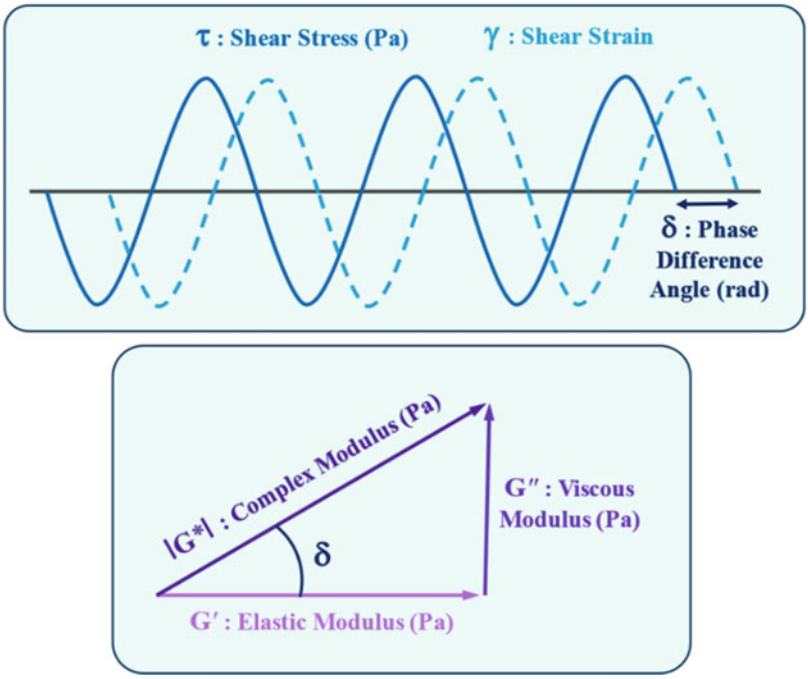

Fig. 2 A schematic of dynamic measurement

which has same phase of strain wave, and the other wave represents the viscous component (τ'') with a phase difference of 90 degrees (Fig. 2).

In an elastic solid, $\delta = 0$ or $\tan(\delta) = 0$ as G' dominates G'' completely. However, in a viscous fluid, $\delta = 90^\circ$ or $\tan(\delta) = \infty$as G'' dominates G' completely. In viscoelastic materials, $0 \leq \tan(\delta) \leq \infty$, depending on the time scale and temperature. When the viscous and the elastic behavior are equal to $\delta = 45^\circ$ or $\tan(\delta) = 1$, the material is making a transition from liquid to solid or the opposite [209–211]. According to Fig. 2, the storage modulus (the energy stored in the material) and the loss modulus (the energy lost in the material) can be described as follows, respectively:

$$G' = \frac{\tau'_0}{\gamma} \tag{3}$$

$$G'' = \frac{\tau''_0}{\gamma} \tag{4}$$

where τ'_0 is the maximum component of elastic stress. Thereafter, the equation for demonstrating the relation between phase difference, storage modulus, and loss modulus would be as follows:

$$\tan(\delta) = \frac{G''}{G'} \tag{5}$$

G'(Pa) and G''(Pa) are usually monitored as a function of time, applied angular frequency, and applied oscillatory strain. In a viscous solution, G'' is greater than G'. Moreover, the complex modulus (G^*) is defined [209] as follows:

$$G^* = \left(G''^2 + G'^2\right)^{0.5} = \mu^* \tag{6}$$

where $\mu^*$is the complex viscosity which can be related to the dynamic viscosity [210]:

$$\mu^* = \left(\mu''^2 + \mu'^2\right)^{0.5} \tag{7}$$

The above equations are recognized as the main relationships to study the rheological behavior of hydrogels.

Rheological tests of protein-based hydrogels can be explained in four categories to present comprehensive and separate characteristics. In general, rheological tests are divided into four parts time sweep tests, frequency sweep test, strain sweep test, and temperature sweep test (Fig. 1).

5.1.1 Time Sweep Test

These tests are applied with aim of start time of the reaction, elastic, viscose, and complex modulus at constant frequencies. Initially and in liquid state, as the reaction has not started, the viscous modulus is larger than the elastic modulus, and also tan (δ) > 1. After a while as the reaction starts of the process of formation of the material network, both the elastic and viscous moduli increase, but the elastic modulus shows a faster growth than the viscous modulus. In other words, the elastic properties transcend the viscous ones [212]. Meanwhile, as reaction continues, at special time at one point, the elastic modulus becomes greater than the viscous modulus (tan (δ) = 1), which results in two-curved collisions [204]. This point indicates the start time of formation of protein-based hydrogel structure [209]. Finally, after the collision point and to the end of the reaction, the value of the elastic modulus is greater than the viscous modulus (tan (δ) < 1) [213]. Besides, the amount of both moduli stay constant while the graph is smooth, indicating the completion of the hydrogel formation process [214]. Figure 3 shows the changes of elastic and viscous moduli of protein-based hydrogels schematically versus time. The transition from liquid to solid state and the gelation of the hydrogel is specified [215]. Moreover, the ultimate value of elastic modulus is an indicator to measure the protein-based hydrogel. For instance, G′ < 10 Pa indicates formation of weak gel which means the hydrogel has low strength. In addition, the gelation time of protein-based hydrogels could support their handling via injection [216]. In order to control the gelation process of the formed soy protein isolate (SPI) hydrogel proverbially and also to clarify the relationship between its property and network structure, the

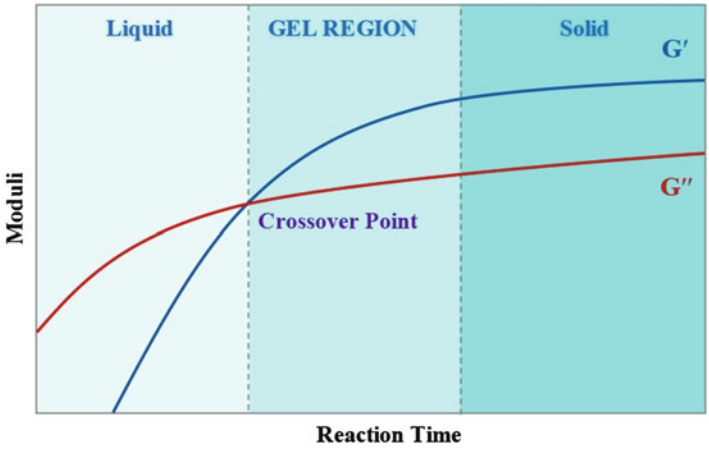

Fig. 3 A schematic of dynamic mechanical behavior along the gelation of the protein-based hydrogel

changes of viscoelastic properties versus time were monitored by the use of dynamic rheometry during the gelation process in the absence and presence of microbial transglutaminase (MTGase) [210]. In the absence of MTGase, the G″ value was larger than the G′ one in the investigated time range showing a dominant viscous property. In this case, gelation time was recorded as long as about 8 h. On the contrary, a crossover point between G′ and G″ was observed in the time range of introducing 0.1 wt % of MTGase into aqueous SPI system which was implied that there was a sol-gel transition. Beyond the crossing, the G′ value becomes larger than the G″ value, indicating that the system becomes more elastic. In this case, the gelation time (*t*gel) and the modulus at the G′/G″ crossover were found to be 15.25 min and 1.65 Pa, respectively [210]. It was found that the *t*gel value decreased with the increase of MTGase amount. The higher the MTGase amount, the shorter the gelation time was. Therefore, it confirmed further that the used MTGase had an obvious catalytic action for the gelation of SPI. For instance, in order to determine the group role of aldehyde at the gelation time of Casein-based hydrogel, time sweep test was used. Based on the crossover point curve, the higher the aldehyde group content or amount of used O-HA, the shorter the gelation time was. In other words, the in situ gelation took place more readily when a higher aldehyde group content or amount of O-HA was used. This may be assigned to the enhanced Schiff's base cross-linking in these cases [204].

5.1.2 Frequency Sweep Test

Frequency sweep tests are the moduli measurement tests as a function of frequency in order to show the hydrogel behavior at short timescales against long ones at constant strain. A critical feature of hydrogel characterization is the dependence of viscous and elastic moduli to frequency. At high frequencies (short timescales), a viscoelastic liquid system can display solid-like behavior, i.e., $G' \gg G''$, while at low

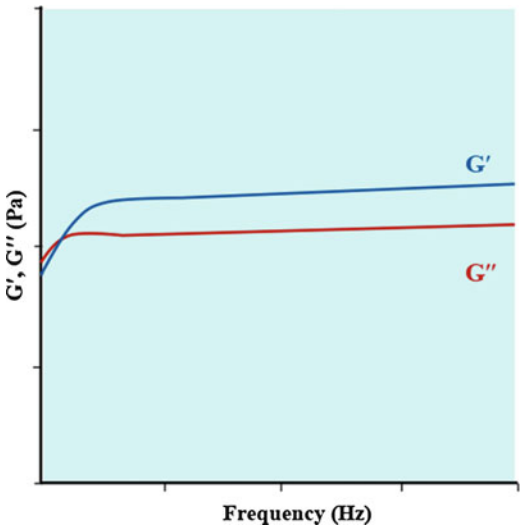

Fig. 4 The elastic and viscous moduli of protein-based hydrogel against the frequency

frequencies (long timescales), it can show liquid-like behavior, i.e., $G'' \gg G'$. Polymeric solutions with a concentration above the entanglement concentration, as well as entangled polymeric melts which are not chemically or physically cross-linked, show $G'' > G'$ (Pa) with a crossover point reached owing to increases in frequency after which $G' > G''$ (Pa). However, a solid, physical hydrogel will display solid-like properties ($G' \gg G''$) at all frequencies and timescales [217]. In order to ensure the formation of hydrogel structure, the elastic modulus is investigated under the frequency changes. The slope of the elastic modulus logarithm in terms of the angular frequency logarithm indicates the network formation of the hydrogel [218]. In other words, the absence of changes in the elastic and viscous moduli versus the frequency approves the formation of hydrogel structure [219]. Also, oscillatory shear tests and the resulting plots of G' and G'' are currently used to demonstrate the gel character, and to classify it as entanglement network or covalently cross-linked gel. It has been shown that covalently cross-linked gels exhibit an elastic modulus greater than the viscous modulus [123]. The illustrated curve in Fig. 4 shows the formation of three-dimensional network of protein-based hydrogel as the solution showed a rheological profile characteristic of elastic behavior with G' highly larger than G'' and almost frequency independent. The plateau storage modulus G'_{∞} value reflects the cross-linker density and strength. Hydrogels with a higher G'_{∞} value can potentially be obtained by increasing the building block concentration and/or using cross-linker proteins with a higher order of multimerization. For instance, G'_{∞} for the engineered disulfide-forming protein and its ligand) Tip1T58C-LE-$(H)_6$ hydrogel (is 262 ± 54 Pa, 15-fold greater than the plateau loss modulus G''_{∞} for the same gel (17 ± 1 Pa), consistent with gel-like materials [220]. Study of the elastic modulus change of SPI hydrogel in presence of MTGase shows that the elastic modulus has only a slight frequency dependence, demonstrating that the resultant SPI hydrogel has a rigid and elastic networks and is

physically stable. The good network stability of the formed SPI hydrogel could be attributed to the chemical cross-linking of SPI in the presence of MTGase, which might be advantageous for its controlled drug delivery and tissue engineering applications [210]. For example, to study the effect of laponite nanoplatelet (LAP) in regenerated silk fibroin (RSF) of *Bombyx mori* silk fiber, G′ and G″ of various RSF/LAP hydrogels as the function of frequency was investigated. It can be seen that the G′ of the RSF/LAP hydrogel increases from about 80 kPa to 200 kPa with the increase of the LAP content from 1% to 5%; and is much larger than that of pure RSF hydrogel (30 kPa). The results suggest that the incorporation of LAP in RSF can greatly improve the mechanical properties of the hydrogel [221].

5.1.3 Strain Sweep Test

An important property of the gel network is that its deformation degree can be determined without inflicting any defects in its network. Accordingly, strain sweep experiments were designed to measure the linear viscoelastic range (LVR) of the hydrogel. The LVR is an area in which no slip occurs between the layers and the hydrogel response is independent of the deformation magnitude while the hydrogel structure is maintained intact (unbroken). In other words, there is a linear relationship between the force applied and the observed deformation. Accordingly, by monitoring the moduli versus strain, the LVR for a given protein-based hydrogel can be determined [222]. The LVR is an outlet of applied strain values while G′ and G″ are independent of applied strain. Linear rheological measurements are classified as studies conducted within the LVR. Unlike LVR, nonlinear rheological measurements are obtained when experiments are performed outside of the LVR. In order to characterize materials in large and rapid deformations and also to have more complete view of responses of soft material against their processing, large-amplitude oscillatory strain (LAOS) measurements are critical, while steady-state shear is used to observe the low of hydrogels. The frequency sweep measurement and the oscillatory strain sweep measurement should be the first measurements to be carried out on the hydrogels during their rheological characterization [139, 223]. An accurate detail of frequency and strain response is important since during an oscillatory time sweep measurement by the rheometer the values of G′ and G″ will be under constant frequency and strains within the LVR condition. As Fig. 5 illustrates, changes in the elastic and viscous moduli of the hydrogel versus the strain changes show a viscoelastic behavior of the material. As it can be observed, at first, the elastic modulus of the hydrogel is higher than its viscous modulus (G′ and G″), and it keeps this superiority, in which this strain reflects the level of hydrogel's deformation (critical strain of the hydrogel). It should be noted that in this region, solid will have solid matter. The more strain increases, the more the hydrogel structure is broken down and the elastic modulus is reduced to less than the viscous modulus. In fact, less than 100% strain, the hydrogel will react to applied external forces and return the tension as much as possible to its original state [139]. To investigate the mechanisms involved in the formation and structure of the SPI hydrogels formed in the absence and presence of MTGase, strain sweep measurements were conducted for the cured hydrogel samples. For all protein concentrations, G′ remained almost constant as

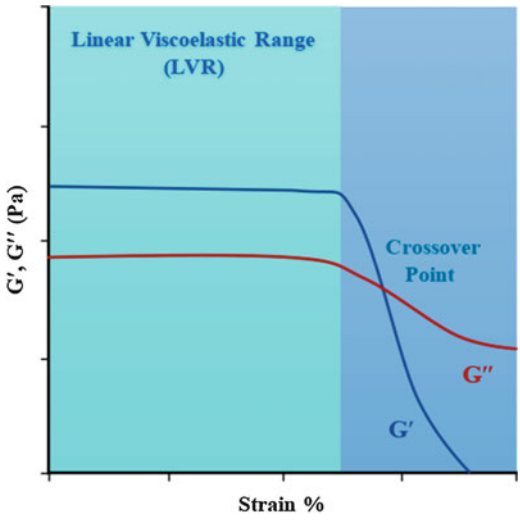

Fig. 5 The elastic and viscous moduli of protein-based hydrogel against the strain

strain increased and then suddenly decreased, indicating the bond breakage within the hydrogel network and a transition from linear to nonlinear behavior. With the increase of SPI concentration, the critical strain of the hydrogel value was found to decrease for the SPI hydrogel formed in the presence of MTGase and increase for the SPI hydrogel formed in the absence of MTGase [210]. For instance, by using strain sweep test, it is indicated that mechanical property of amino acid-based super-absorbent polymer hydrogel can be improved by copolymerization with flexible 2-(2-methoxyethoxy)ethyl methacrylate (MEO2MA) comonomer [224].

5.1.4 Temperature Sweep Test

Regarding the dependence of the hydrogel gelation time (the start time of the reaction) to temperature and the importance of temperature in the application of protein-based hydrogel, the investigation of the reaction start temperature, its effect on the hydrogel gelation time, and the limitation of the applied hydrogel temperature is considered essentially. Moreover, using this method shows the information related to the melting temperature indicating that the reaction is thermoreversible or not [103]. For this purpose, to maintain the three-dimensional structure of hydrogel, modifications of the elastic, viscous modulus, or complex modulus must be considered for the temperature of the hydrogels [219, 225]. It should be noted that the rate of increase in temperature in the rheometer, depending on the type of operational environment of the protein-based hydrogel, is applied to the hydrogel. In addition, the hydrogel gelation time can be determined versus time and temperature by checking the complex viscosity against time at various temperatures. The sudden increase point in viscosity represents the beginning of the gelation. In Fig. 6, the hydrogel state from viscous fluid to viscoelastic solids is illustrated versus temperature. For instance, in Fig. 6a, the collision point of the elastic and viscous modulus,

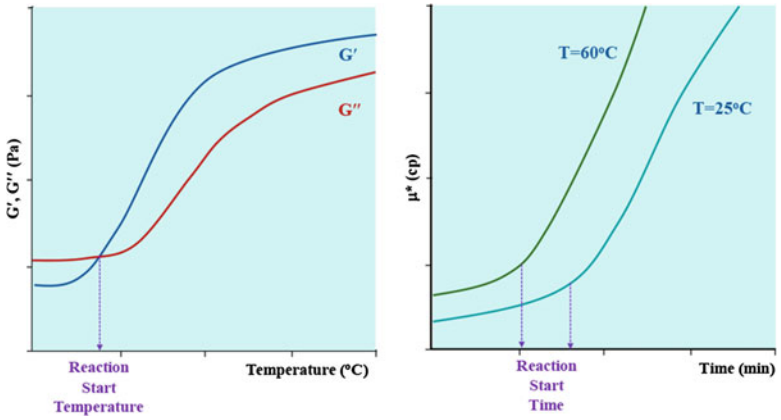

Fig. 6 The elastic and viscous moduli variation of protein-based hydrogel in face of time and temperature

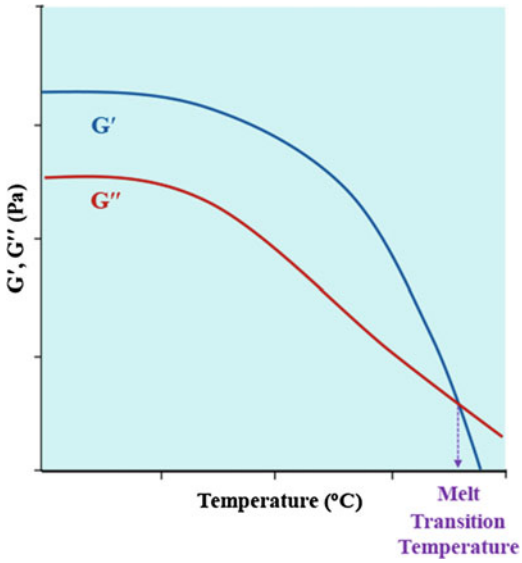

Fig. 7 The elastic and viscous moduli of protein-based hydrogel versus temperature

and the increase of G′ to G″, represents the reaction start temperature. Also in Fig. 6b, variations in the complex viscosity versus time at different temperatures indicate the endothermic reaction of the protein-based hydrogel of that example.

Figure 7 illustrate the modulus variation among temperature for the viscoelastic hydrogel. The melt transition temperature is assumed as the crossover point of both components of the dynamic modulus, or the sudden decrease in complex modulus G*.

5.2 Morphology Tests

5.2.1 Microscopic Analyses

The hydrogel pore size directly affects the rate of mass transfer between the hydrogel interior and the surroundings and is therefore a critical consideration for applications such as tissue engineering and biocatalysis [220]. In addition, a three-dimensional network can be detected using a scanning electron microscopy (SEM) image [11, 202, 206]. Therefore, investigation of the effect of different methods in the process of hydrogel synthesis and the size of the created pores in the porous structure would be possible by using micrograph images. Besides, the energy dispersive spectrometer (EDS) accessory can be also used to detect and determine a few elements in the hydrogel [221].

It should be noted that transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM) are two similar techniques that image the electron beam from the specimen [103]. TEM and STEM versus SEM have higher spatial resolution and additional analytical measurements. However, in these two methods, more precision is needed to build the sample [226, 227]. Moreover, the atomic force microscopy (AFM) test can also be used in the presence of nanomaterial in the structure of protein-based hydrogel to ensure nanoplatelets well disperse in the hydrogel matrix [93, 221].

5.2.2 Other Tests for Detecting and Characterizing the Protein–Based Hydrogel

The molecular structure of hydrogels has mainly been investigated using spectroscopic methods. Analysis of the polymers has been carried out using infrared spectroscopy, proton nuclear magnetic resonance (NMR) spectroscopy [108, 222]. Regarding the fact that the studied hydrogel of this study is protein-based, the presence of amide bonds within the hydrogel was expected. Therefore, Fourier transform infrared spectroscopy (FTIR) test is used as a suitable technique for protein-based hydrogel analysis [103]. An infrared spectrum represents the fingerprint of a protein-based hydrogel sample. Thus, infrared spectroscopy can be efficient in better identification (qualitative analysis) of hydrogel types. In addition, based on the size of the peaks in the spectrum, the amount of material available in the structure of the hydrogel can be investigated. Also, thermo-responsiveness of hydrogels was studied using a UV–Vis spectrometer equipped with a temperature controller [224].

5.3 Thermal Stability Analysis

The thermal analysis of the protein-based hydrogels was performed under differential scanning calorimeter (DSC) or thermal gravimetric analysis (TGA) [24, 82, 103, 180]. In order to measure the structural strength of protein-based hydrogel against temperature increase [130], the structural changes of the hydrogel to the temperature increase can be examined relatively with controlled state. In addition, the melting temperature and glass transition temperature are also measurable by this method.

6 Conclusions

All proteins have a potential to form hydrogel due to the presence of functional groups such as amino and carboxyl in their structure for cross-linking. Nowadays, several proteins and peptides have been used to obtain hydrogels. Collagen and gelatin are the most studied ones for hydrogel preparation. Physical, chemical, or enzymatic treatments can be applied to cross-link protein, which results in hydrogel formation. The preparation methods mainly depend on hydrogel applications. Physical hydrogels are reversible and sensitive to environmental conditions while chemically cross-linking causes stable structure in hydrogels. Glutaraldehyde is the most popular agent for chemical cross-linking, although it is toxic to living cells. Therefore, enzymatic cross-linking particularly using transglutaminase is preferred to obtain biocompatible hydrogels.

To develop protein-based superabsorbent hydrogels, proteins originated from soy, canola, cottonseed and chicken feather, and fish processing industries as well as zein and even collagen and gelation may be proper choices because of their low cost. Casein and egg white albumin seem to be used attractively in food industries for nutrient encapsulations. The other sources like silk, keratin, elastin, resilin, peptides and also collagen and gelation, showing proper biocompatibility, are applied in biomedical engineering. Sometimes, proteins should be chemically modified to obtain hydrophilic hydrogels and increase water absorbency.

Proteins can be employed individually or in combination with the other polymers and minerals, to develop hybrid and composite hydrogels, respectively. Hybridizing and compositing insert several features to hydrogels. For example, water holding capacity, mechanical stability, and rheological behavior are highly influenced by such combinations.

For practical applications, performing the rheological tests to find out the structural and thermal strengths, the effect of compositions on viscoelastic behavior, critical strain of protein-based hydrogels could be directive.

However, environmental challenges and the needs for sustainable development enforce the use of renewable, biodegradable, and biocompatible hydrogels. Proteins are a proper alternative for developing such hydrogels.

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