JFET 27(1) (2023) 48-63

Journal of Faculty of Engineering & Technology

journal homepage: www.pu.edu.pk/journals/index.php/jfet/index

A state-of-the-art Review on Fabrication and Characterization of Biopolymer-based Hydrogels

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Abstract

Hydrogels are three-dimensional networks that can hold huge amounts of water while maintaining structural integrity. Hydrogel matrices are manufactured by blending biopolymers, like natural and synthetic polymers, proteins, polysaccharides, or their derivatives, via incorporating crosslinking agents. These mediums give a biocompatible, environmentally benign substitute to synthetic hydrogels. The generation of biopolymers begins after detailed research of numerous preliminary measures such as biopolymer type, concentration, and crosslinking density. Swelling studies, FTIR, GC, HPLC, NMR, AFM, TGA, and MS were used to assess behavior of freshly prepared hydrogels. Swelling experiments indicate high retention of water and respond to external stimuli such as pH and temperature variations. Mechanical experiments reveal viscoelastic properties of hydrogels, such as compressive and tensile strength, representing their potential for load-bearing applications. Further, the microstructure of hydrogels is investigated using SEM, which provides information on their porosity, pore size distribution, and surface shape. Drug release studies indicate that these hydrogels have the potential to be employed as drug delivery vehicles with adjustable release rates.

Keywords: Biopolymers, Hydrogels, Cross-linkers, Swelling studies, Drug release

1.1. Introduction

Many struggles have been made in the current era to induce nature-stimulated substances that comprise qualities necessary for biomedical applications. Hydrogels, which are 3-D networks of hydrophilic polymers cross-linked via chemical or physical reactions, have attained recognition under different names such as smart hydrogels, 3D hydrogels and aqua gels. Hydrogels own an extremely porous, interconnected, hydrophilic, and cross-linked polymeric chain and engross and store significant amounts of water or other fluids (>20%) deprived of degrading the basic structure of polymer. The chemical reaction between polymeric networks and H_2O or biological solutions occurs via capillary, osmotic and hydration forces leading to extension in polymer network [1]. Hydrogels offer unique features, such as extraordinary absorptive activity in polluted water. Because of ability of polymers to be cross-linked with one another, they may retain a considerable amount of water or any aqueous solution [2]. Homo-polymers or co-polymers possess network chains that are insoluble because of the incidence of chemical or physical crosslinks, such as

participation or crystallization. The high water retention capacity of materials makes them biocompatible [3]. Because of the huge water content, these are flexible like earthy tissues.

Hydrogels have thermodynamic properties with water that allow them to swell in the supplied medium. Hydrogels are recognized as good natural absorbents since they contain a large volume of water (over 90%). In modern times, numerous varieties of hydrogels have been produced, comprising of environment-responsive, self-healing, self-assembled conductive, shape memory and super-molecular hydrogels, and their properties are sightseen and tested in various applications [4]. In current decade, several types of hydrogels have been produced, including environment-responsive, self-healing, self-assembled conductive, shape memory, and super-molecular hydrogels, and their properties are being explored and evaluated in various applications [5]. This behavior found in organisms has inspired formulation of various kinds of hydrogels. When a microstructure is destroyed, specific physicochemical interactions occur following reaction criteria and thus, biological substances amend themselves and reinstate their real assignments. Novel intelligent hydrogels may be formulated by blending three-dimensional hydrogels with self-healing capability. Self-healing hydrogels are characterized in two classes: 1) autonomic self-healing hydrogels and 2) non-autonomic self-healing hydrogels. There is no need of stimulus for autonomic self-healing hydrogels for their reconstruction because destruction controls restoration. The remedial process begins by applying stimuli like the magnetic field, light and optimum temperature [6] [7]. The harmonious behavior of hydrogels in polymer formulations, copolymerization, and combination processes is utilized to prepare various new hydrogels together with topological gels, nanocomposite gels, double network gels, and polymerization motor gels [8]. Hydrogels are gifted ingredients for slightly aggressive conduct of cartilage abrasions as these can mimic the swelling and mechanical performance of strong tissues, that are chiefly serene of extracellular matrix (ECM) comprising of collagen and glycosaminoglycan and are characterized because of having huge amounts of water that allows to allocate stress homogeneously across specific area [9]. These are laboring in biomedical fields such as sustainable and control release of drugs, wound healing, drug delivery, and wound dressing owing to their superior qualities such as biocompatibility and their ability to impersonate several aspects of genuine tissue [10]. Hydrogels perform a symphony of roles in the biomedical orchestra. Their critical function in medicine delivery is at the forefront. Consider a scenario in which pharmaceuticals are delivered precisely to provide maximal therapeutic outcomes while minimizing negative effects. With their customizable features, hydrogels set the way for continuous and tailored medication delivery, rewriting the patient care narrative. Hydrogels have achieved huge fame due to their lower volume fraction, large capacity of water retention and uses in many applications like scaffolding in tissue engineering, biomedical devices, wound dressing and wound healing, cartilage retention [11], pharmacology and drug delivery [12]. In this review article synthesis and gelling methods of biopolymer-based hydrogels and their characterization techniques are described.

Hydrogel fabrication and characterization are difficult processes, and researchers are always working to overcome numerous obstacles and research gaps in these fields. Here are some typical issues and research gaps in hydrogel manufacturing and characterization. Controlling the microstructure, porosity, and pore size distribution of hydrogels remains difficult. Hydrogels with customized structures for specific uses, such as tissue engineering or drug delivery, are frequently difficult to fabricate. Some hydrogel production processes or components may cause cytotoxicity or biocompatibility concerns. It is vital to ensure that hydrogel synthesis procedures are safe for usage in biological systems. It is difficult to achieve repeatability in hydrogel synthesis, especially when moving from lab-scale to large-scale production. The development of scalable and repeatable production procedures is critical for moving hydrogel innovations from bench to bedside. It is a constant challenge to include bioactive compounds, such as growth factors or

medicines, into hydrogels without compromising their stability or activity. This is especially relevant for medication delivery and regenerative medicine applications. Printing hydrogels with high fidelity while retaining cell viability throughout the process is difficult in 3D bioprinting. Improving the printability and resolution of hydrogel-based bio-inks is a work in progress.

1.2. Polymers

The foundation of polymers is the repetition of basic structural monomers to build a three-dimensional construction and occurs in all biological systems either hydrophilic or hydrophobic. The hydrophilic mechanisms expand the hydrogel, while hydrophobic constituents regulate swelling ratio and enhance gel's mechanical qualities. The existence of hydrophobic components distinguishes temperature-sensitive gels, while hydrogel swelling and deswelling may be regulated via altering the ratios of hydrophilic-to-hydrophobic groups [13]. These groupings are capable of full dissociation as well. Hydrogels are classified as super-absorbent; hence, they can absorb enormous amounts of water or solvent used for their formulation. The stimulus and elastic restoring force of polymer chain exert osmotic pressure to determine the quantity of water absorbed by hydrogels. Water-soluble (synthetic, semisynthetic, and natural) polymers are utilized to create hydrogels [14]. Humans have been using natural polymers such as horn, hair, and cellulose since beginning [15]. The polymers synthesized by humans are named as man-made synthetic polymers and are nearly as numerous as natural polymers, while majority of advancements in this development began during the Second World War. Synthetic hydrophilic polymers (hydrogels) are a kind of hydrogel that is formulated by chemically stabilizing hydrophilic polymers in a 3D network. Synthetic polymers have received huge popularity in both technological and medicinal applications.

When crosslinking between polymer chains is done, resulting networks display viscoelastic behavior. The major characteristic of hydrogels is that crosslinker quantity may be regulated. Additionally, stimulus responsive substances are created via incorporation of a cross-linker with specified properties. By radical polymerization of vinyl monomer combinations, chemically cross-linked hydrogels are generated [16].

The polymers having large capacity to retain water is because of existence of functional groups (OH, COOH, and NH_2) is a keynote for formulation of hydrogels. Polymers interact with functional groups that have complimentary reactivity, like amine-carboxylic acid or isocyanate-OH/NH2 reactions, through Schiff base reagents, and form covalent bonds. Additionally, crosslinking by condensation reactions, addition reactions, high energy irradiation, and enzyme crosslinking is also described for chemical hydrogels.

Alginate is a renowned polymer, able to do cross-linking by following ionic chemical combinations. It is a polysaccharide that may be crosslinked by Ca^{2+} ions and comprises mannuronic and glucuronic acid residues. The cross-linking capability of alginate is accomplished at ambient temperature and pH levels. Consequently, these hydrogels are usually utilized as a medium for encapsulating live cells and releasing proteins. The gels are destabilized by chelating agents that remove Ca^{2+} ions from the gel. When sodium alginate solution is sprayed in $CaCl_2$ solution, the microparticles of alginate release a protein and this process is regulated by glazing particles with polymers having cationic chains like chitosan and polylysine [17].

1.3. Biopolymers

Biopolymers are polymers that originated naturally during the growth cycles of all creatures and are biodegradable. Biopolymers are natural polymers that can be synthesized biologically from living organisms such as algae or chemically manufactured from biological components. Therefore, biopolymers coin the term natural polymers. There are various metabolic systems necessary for life in living organisms generate such as natural polymers. Living material can

create a vast variety of polymer species, such biopolymers account for most cellular dry matter. Such renewable resources stimulate awareness of pharmaceutical, biomedical, cosmetic, and food industries owing to their chemical composition, physical behavior, extensive diversity, and are cheaper. Cellulose, collagen, fibrin, hyaluronic acid, curdlan, pullulan, carrageenan, chitosan and starch are the most fascinating materials for different applications [18]. Various classes of biodegradable polymers have been proposed based on the development of the synthesis. Biopolymer activities are, in most environments, vital for cells and are as diverse as their forms. Biopolymers or natural polymers functionalize their role in catalysis of chemical reactions, swapping of genetic material, protection from unwanted or harmful substances, environmental safety related to biotic and abiotic elements and intervention of the adhesiveness to surfaces of different living organisms or of non-living materials. Biopolymers are prepared via enzymatic procedures in cytoplasm, in numerous sections or organelles of cells, at the cytoplasmic membrane or at cell wall mechanisms, on exterior portion of cells, blending of biopolymers can also originate in a chunk of a cell or sustainable in alternate portions as it happens [19]. Biopolymers are formulated in various ways. Many biopolymers are found in profusion naturally. So, these are extracted from plants and algae that thrive in nature. Agar and alginates are derived from red algae of the species Gelidium or from other brown algae, often known as seaweeds. Carrageenan is obtained from red algae like *Kappaphycus alvarezii*, *Hypnea musciformis* and *Chondrus crispus.* Some biopolymers are extracted naturally using natural sources. One notable exclusion is hyaluronic acid, which is taken from newborn children's umbilical cords. Biopolymers can be produced in vitro using isolated enzymes in cell-free environments. Fermentative production of biopolymers, such as polysaccharides, is employed in industry. The biotechnological manufacturing of biopolymers can be either intracellularly or extracellularly. Biopolymers have been employed in the food and cosmetic industries for more than 10 years, and they have also been researched in pharmaceutical applications as functional receivers, bioactive components, and in tissue engineering [20].

1.4. Blending of polymers

Polymers blending is not only the process of mixing two or more polymers or copolymers to synthesize novel materials but a powerful tool for creating new hydrogels with improved or unique characteristics. Polymer combinations and composites are very resourceful to meet novel requirements for various material qualities [21]. Polymer blending can be established using numerous techniques like melt blending, solution mixing, and in situ polymerization. The extensively used method for polymer blending is melt blended in which two or more polymers are mixed in a single screw or twin-screw extruder. The temperature of melt is lowered down and put it to dry to be converted into a hydrogel. The other most widely used method is solution blending which includes dissolution of two or more polymers in a solvent and a homogenous mixture is prepared. The solution is then evaporated to remove the solvent and form a new substance. In situ polymerization is a method in which two or more monomers are polymerized that creates a copolymer. Polymer blending is extensively used in industries. One of the main reasons is to improve mechanical characteristics of a polymer like strength, toughness, and flexibility. Another reason is to adjust chemical and physical properties of a polymer, for example its thermal stability, resistance to UV light and adhesion to different substrates.

1.5. Classification of hydrogels

Hydrogels are grouped as per their physical characteristics, swelling behavior, mechanism of synthesis, source of occurrence, ionic charges, rate of biodegradation, and kind of crosslinking used in practice. The crosslinking process in physical gels is physical in nature. Hydrogels are typically created by physical mechanisms such as hydrophobic association, chain aggregation, crystallization, polymer chain complexion, and hydrogen bonding. To create a chemical hydrogel,

a chemical procedure called chemical covalent crosslinking (simultaneous or post polymerization) is adapted. Because of stereotype, former hydrogels can be reversed, but later are permanent and irreversible owing to configurational alterations [22, 23].

The stimuli-responsive swelling hydrogels are ecologically friendly and have a high capacity to adapt alterations in their external environment [24]. In response to differences in ionic bonding or pH of the nearby biological fluid or temperature, they display various intricate modifications in their swelling qualities and polymer network structure. Because of their composition, these chemicals have a wide range of uses, including segregation of membranes, chemical valves, and certain medication delivery systems. Gelation deals with linking of macromolecular network collectively, that formerly leads to gradual heavier branching; until now, soluble polymers are dependent on the structure and composition of the beginning components. Stimuli-responsive polymers have gained popularity owing to their capability to imitate respond mechanism of living systems. A few alterations in environmental stimulus can cause separate intermediate changes in physicochemical characteristics. Temperature-, pH-, photo-, electro-, and multi-responsive polymers can be classified constructed on their response to different stimuli. Several constituents based on these "intelligent polymers" are now being developed and employed in biomedical applications like drug delivery systems, wound healing, soft robotics, tissue engineering, bio-separation, and biosensor design. Synthetic biodegradable polymer-based products drew the most interest due to their promise in vivo uses. As a result, creating simple and effective ways for synthesis have changed biopolymers to give intelligent activities and it is significant for [25] the advancement of biomedical materials [26-28]. The response to ligands, enzymes, antigens, and various biochemical agents constitutes a biochemical stimulus.

Polymeric hydrogels containing ionic pendant groups were discovered by scientists to receive or share protons to correspond to pH variations in environment. pH sensitive hydrogels are divided as anionic or cationic. Anionic hydrogels include pendants like carboxylic or sulfonic acid, and deprotonation happens upon exceeding of pH, thus, resulting in ionization of the pendent groups and enhanced capacity of swelling of hydrogels. The polymer characteristics, such as concentration, density of cross-linking materials, ionic charge of ionizable groups, hydrophilicity or hydrophobicity, and degree of ionization, come under initial consideration. The 2nd constituent is swelling medium's parameters for example pH, ionic strength, counterion, and polyvinyl sulfonic acid valency (PVSA) [29].

The structure and composition of thermo-reversible hydrogels are comparable to the structures and compositions of negative and positive temperature hydrogels. The distinction between these and the preceding two forms of thermo-sensitive hydrogels is in the sort of chemical linkage found in them. The hydrogel shares a sol-gel phase intermediate state rather than a swelling-shrinking transition because polymers in this class are not covalently bonded. Instead of sol-gel transitions, volume changes occur in chemically crosslinked thermosensitive hydrogels. There are various chemical reactions like hydrophobic terminal points and hydrogen bonding that suddenly shift volume at the critical solution temperature (CST). In the swollen stage, water molecules create hydrogen bonds with polar groups of the polymer chain within the hydrogels, forming an iceberg of water that surrounds the hydrophobic groups. At the CST, hydrogen bonding among polymer and aqueous solvents has become uncomplimentary when compared with polymer-polymer and water-water interactions, forcing rapid desiccation of the system and removal of water from gel and increasing its entropy, results in contraction of the polymeric chains [30].

The most important class of hydrogels is thermally reversible hydrogels that experience converting from sol to gel on action of stimuli. Reverse thermal gelation can cross-link polymers with hydrophobic realms in aqueous conditions, when the hydrophobic section is joined to hydrophilic end by grafting or copolymerization, amphiphiles are soluble in water at lower

temperatures [31]. An increase in entropy of added solvent is observed with rise in temperature because hydrophobic terminals gather to decrease surface area of solvent. The concentration and chemical composition of polymers, hydrophilicity and hydrophobicity determine the temperature for gelation. Pluronics and tetronics, frequently employed thermo-reversible hydrogels recognized by the food and drug administration (FDA) and environmental protection agency (EPA) for usage in food products, medicinal compounds, and agricultural goods were reported by Li et al [32].

1.6. Synthesis of hydrogels

There are various methods for synthesizing biopolymer-based hydrogels, including physical and chemical crosslinking. Physical crosslinking includes methods such as thermal or photo-crosslinking, whereas in chemical crosslinking, crosslinking agents such as glutaraldehyde, genipin, and carbodiimides are being added. Biopolymers like alginate, chitosan, collagen, gelatin, and hyaluronic acid are enormously used to synthesize hydrogels. It has been observed that the introduction of co-monomers such as acrylic acid and acrylamide improves mechanical characteristics of biopolymer-based hydrogels.

A biopolymer blend and a cross-linking agent is heated before formation of gel to induce cross-linking. Thermal cross-linking is done to synthesize biopolymer-based hydrogels, and it commonly consists of four phases. The initial stage is preparation of biopolymer solution. The biopolymer is dissolved in an appropriate solvent, such as water or an aqueous buffer, and a homogeneous solution is formed. The concentration of biopolymer blends may vary to attain the desired characteristics of the freshly formed hydrogel. After that cross-linking agent is incorporated. Crosslinking agents such as vinyl trimethoxy silane, polyethylene glycol, glutaraldehyde, or genipin are added to the biopolymer solution. The crosslinking agent forms biopolymer chains, generating covalent bonds among them and thus, creating a formulation of a hydrogel web. The next phase in hydrogel synthesis is heating of prepared blend. The prepared blend with cross-linker is then heated to initiate crosslinking before achieving desired temperature for gelation. The gelation temperature is that temperature at which biopolymer solution alters its state from a liquid to a gel-like structure. The fourth and final step is cooling. After the crosslinking is done, the reaction is assumed to be complete, the hydrogel is cooled at room temperature to permit it to settle down and stabilize itself. The characteristics of resulting hydrogel are adjustable to amount of biopolymer solution, its type and concentration of the cross-linking agent, and heating and cooling parameters [33].

Photo-crosslinking encompasses usage of UV or visible light to develop crosslinking of biopolymers. Photo-crosslinking is achieved by adding a photo-initiator like UV radiation into polymer solution, that can be stimulated by subjecting to light. The photo-initiator is a substance that generates free radicals or other reactive species when exposed to light and initiates crosslinking between polymer networks. The light source can be a UV or visible light source depending on the type of photo-initiator used. The photo-crosslinking of hydrogels incorporates UV/Visible light to begin cross-linking amid polymer chains. A biopolymer is liquified in an appropriate solvent to homogenize the solution. A photo-initiator incorporates polymer solution. The bombardment of light radiation of suitable wavelength and intensity for time is done on both polymer solution and photo-initiator. The light stimulates photo-initiator, creating cations or anions that recruit cross-linking between the polymer networks. The next step is termed as post cross-linking treatment. In this step any unreacted species or residuals of remaining photo-initiator are detached by washing hydrogel with some solvent. Photo-crosslinking is advantageous for hydrogel synthesis, involving spatial orientation and speed of gelation. This technique is also helpful for employment of complex structures, removal of toxic chemicals, thus, considering a necessary method for biomedical uses [34].

Ionic gelation incorporates collaboration of charged biopolymer ions with to prepare a hydrogel network. For example, alginate hydrogels can be manufactured by mingling an alginate solution with a divalent cation solution, for example $CaCl_2$. A biopolymer is dissolved in a right solvent to prepare a homogenous mixture. The amount of polymer solution depends upon obligatory characteristics of hydrogel. A crosslinking agent, such as a divalent metal ion solution, is added to the polymer solution. The crosslinking agent cooperates with charged groups on polymeric sides, generating ionic bonds, and crosslinking chains. Crosslinking reactions happen naturally or under specific parameters, for example pH or temperature alteration, during gel formation, following the creation of a hydrogel. After this, the hydrogel is washed with a solvent to eradicate any unreacted species or pollutants and then dried by putting in oven. Ionic gelation is a simple and flexible process for preparation of hydrogels, and it is frequently employed to formulate alginate and chitosan-based gels [35, 36].

Another effectual technique to prepare hydrogels from biopolymer solution by incorporating nanofibers is electrospinning. These nanofibers crosslink and hydrogel chain are formed. A biopolymer blend is prepared and applied current. The sample is loaded into a syringe comprising of a fine nozzle. As the solution is ejected via nozzle, the electric field spreads polymer solution into fine fibers, and are gathered on a grounded collector. The collected fibrous scaffold is then cross-linked via physical or chemical cross-linking method to synthesize a hydrogel. The characteristics of prepared hydrogel are adjustable to amount of the polymer solution, the electrospinning conditions such as applied voltage and flow rate, and the crosslinking parameters. It is a promising technique for formulating hydrogels as it allows for construction of a fibrous scaffold with a huge surface area to volume ratio, that can augment cell attachment and propagation. These hydrogels are potentially applicable to cure problems in tissue engineering, drug delivery systems, and wound healing [37, 38].

Freeze-thawing is another physical procedure for manufacturing hydrogels that includes repetitive freezing and thawing of a polymer solution to persuade cross-linking among the polymer networks. The biopolymer is dissolved in a preferable solvent and a homogenous blend is prepared. The polymer solution is kept at lower temperature for frozen purpose characteristically less than -20°C, consequently forming ice crystals. The frozen solution is kept on thawing at room temperature, and thus, preparing a hydrogel. After this hydrogel is subjected to multiple freeze-thaw cycles, normally between 3 to 5 cycles, to further increase capacity of crosslinking, thus, improving mechanical properties of hydrogel. The characteristic of resulting hydrogel is adjustable by altering concentration of polymer solution, number of freeze-thaw cycles, and freezing and thawing conditions. Freeze-thawing is an easy and cheaper technique for synthesizing hydrogels and is commonly opted to synthesize collagen-based hydrogels. The resulting hydrogels are biocompatible and have excellent swelling properties, making them suitable for use in biomedical applications like tissue engineering and drug delivery [39, 40].

These physical approaches for biopolymer-based hydrogel synthesis have various advantages, including simplicity, gentle reaction parameters and absence of harmful ingredients. However, as compared to chemically crosslinked hydrogels, the qualities of the resultant hydrogels, like mechanical strength and porosity, might be restricted. There are numerous chemical procedures for formulation of hydrogels to overwhelm these limitations. Free-radical polymerization, cross-linking of pre-formed polymers, electrostatic assembly and thiol-ene click chemistry are the most used chemical methods to formulate hydrogels.

Free radical polymerization is an extensively employed technique. The monomers like acrylic acid, acrylamide, and N-vinyl pyrrolidone are selected for polymerization. Crosslinking agents such as ethylene glycol dimethacrylate (EGDMA) and N,N'-methylenebis(acrylamide) (MBA) are added to create covalent linkage among polymeric chains, resulting in a 3D network. Free radicals

are introduced for starting polymerization. Ammonium persulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED) are widely used initiators. The monomers, crosslinking agent, initiator, and solvent (typically water) are blended and polymerized. The unreacted monomers and any pollutants are removed by washing the freshly prepared hydrogel with water.

Hydrogels are prepared by crosslinking of pre-formed polymers [41]. Crosslinking is of three types, 1) physical, 2) chemical, 3) enzymatic means. Physical crosslinking states to establish reversible reactions amid polymer chains, for example, hydrogen bonding or electrostatic interactions. Chemical crosslinking includes covalent bond between polymer chains incorporating crosslinking chemicals like glutaraldehyde or ethylene glycol diglycidyl ether. The employment of enzymes to catalyze covalent connections between polymer chains.

Several strategies for preparation of cross-linked hydrogels rely on free radical reactions. The initial step is a copolymerization-cross-linking reaction that incorporates either one or more than two monomers and one multifunctional monomer, both of which exist in traces. This type of formulation is termed as free radical polymerization and they are activated by decomposing peroxides or azo compounds, as well as application of ionizing radiation or UV light. The electron stream of light, gamma rays, or X-rays, ionizing radiation methods ignite a polymer and generate a cross-linked structure by following free radical mechanisms. Such reactions, that frequently happen without oxygen or air, may lead to quick formation of a 3-D network. Chemical cross-linking requires a linear or branched polymer to cooperate directly with a di-functional or multifunctional, low-molecular-weight cross-linking agent. This mediator often joins two higher-molecular-weight chains through di- or multifunctional groups.

A comparable process applies the interaction of a small dual functional fragment with linear polymeric network that includes pendant or terminal reactive groups such as -OH, -NH2, NCO, or -COOH, and the bi-functional molecule cross-links the chains. Natural polymers, like proteins, can also be cross-linked in alike pattern via utilization of enzymes. Transglutaminase, for instance, cross-links interaction of protein glutamine amide groups with lysine amino groups. The below mentioned reaction explains this process.

$$- (CH_2)3 – CONH_2 + - (CH_2)4 – NH_2 + \text{transglutaminase} \rightarrow - (CH_2)3 - CONH - (CH_2)4 - + NH3$$

The other enzyme-catalyzed cross-linking method involves hydroxyphenylpropionic acid (tyramine) was conjugated to gelatin and cross-linked to produce a hydrogel via an oxidation reaction with hydrogen peroxide (H_2O_2) catalyzed by horseradish peroxidase (HRP) [42, 43].

The method of creating hydrogels on an electrode surface is commonly referred to as electrochemical hydrogel manufacturing. Yet, additional terms for this technique include electro-deposition, bio-assembly, bio-printing, e-gels, and electro-gelation. Hydrogels may be generated by altering solubility of the gelatin constituent on surface of electrode [44]. To generate this shift in solubility, several reduction and oxidation (redox) procedures are utilized. Typically, techniques are determined based on a blend of the gelator kind, solution composition, and anticipated product attributes. Though these approaches for producing hydrogels on an electrode surface vary, basic rules remain the same. Generally, gelator dissolves in an increased amount of solution but unable to deposit at the electrode surface, thus, creates solubility gradient. Because of alteration levels of solubility, gelatin constituents combine to form hydrogels on electrode surface although residual soluble in the bulk solution. A working electrode, for instance glassy carbon, platinum, or FTO/ITO (fluorine doped tin oxide) coated glass, is utilized in a three-electrode system. Electrode surfaces may be customized to produce a precise form of hydrogel. Electrode pens are small and portable, and they may be used to plan areas inside a bulk gelatin solution. Gelation using an electrode pen offers more 3-D regulator inside a bulk solution rather than typical triggers. An electrode pen attached to a mechanical arm enables programmable 3-dimensional printing of hydrogels [45].

Electrostatic assembly is a process used for preparation of hydrogels by assembling oppositely charged polyelectrolytes. This method involves the layer-by-layer deposition of polyelectrolytes on a surface, where each layer is formed by electrostatic attraction between positively and negatively charged polyelectrolytes. The resulting hydrogel consists of a multilayered structure of polymer chains that can captivate and hold bulky quantities of water. The first step is to select the polyelectrolytes to be used in the electrostatic assembly. Polyelectrolytes such as Poly (acrylic acid) (PAA) and poly (allylamine hydrochloride) (PAH) contain ionizable functional groups, for example, carboxylic acid or amine groups. Polyelectrolytes are charged after dissolving in aqueous solution with specific pH. The concentration of the polyelectrolyte solution influences stability and thickness of gel. The polyelectrolyte solution is coated on a surface, like a glass slide or a nanoparticle by dipping or spraying [46, 47].

Photopolymerization is a technique to prepare hydrogels in which smaller monomers are polymerized via photochemical reaction using a catalyst to create a polymeric network [48]. Catalyst can be a source of ultraviolet or any chemical. An appropriate solvent is required to initiate photopolymerization. As hydrogels are highly cross-linked, and exhibit have high swelling capacity, therefore, this is a challenge for this process of synthesis [49].

1.7. Characterization of hydrogels

The morphology of hydrogels may be examined using a scanning electron microscope [50] and transmission electron microscopy, that elucidates information about the structure and organization of the hydrogel network [51]. In-vitro biocompatibility studies are performed to evaluate the cytotoxicity and tissue response to the hydrogels. Biopolymer-based hydrogels are utilized as drug delivery vehicles. The release kinetics of drugs from hydrogels is evaluated using technical apparatus like UV/Vis spectroscopy [52, 53], high performance liquid chromatography [53] and fluorescence spectroscopy. Biopolymer-based hydrogels are biodegradable, and the rate of degradation is a significant parameter for many applications. The degradation behavior can be evaluated using techniques such as gravimetric analysis, gel permission chromatography [54] and Fourier transform infrared spectroscopy. Hydrogels are also characterized by using XRD, differential scanning calorimetry, cytotoxicity, antimicrobial, atomic force microscope, thermogravimetric analysis, permeate flux, salt rejection, membrane hydraulic resistance, impedance spectroscopy, mechanical testing, dynamic mechanical analysis, universal testing machine and rheological analysis [55].

1.7.1. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is an influential technique for depicting chemical composition and structure of hydrogels. FTIR spectroscopy works by calculating the absorption of IR rays by the functional groups present in the hydrogel. Different functional groups have distinguishing absorption frequencies that are used to recognize and quantify them. FTIR spectra are typically attained by passing IR radiation via a sample and measuring quantity of radiation absorbed at each frequency.

The FTIR spectrum has several properties to check functional groups in hydrogels. One prominent feature is the presence of characteristic peaks that associate to numerous functional groups in the hydrogel. For example, the peak at about 1700 cm^{-1} resembles the carbonyl group (C=O), which is present in various hydrogel-formulating monomers, for example acrylamide. Cellulose displays peaks at 1200, 1050, and 900 cm^{-1}, showing C-O stretching, C-O-C stretching, and β-glycosidic linkage, respectively. The intensity and form of the peaks are associated with water molecules found in hydrogels. Water molecules have distinctive peaks at 3200, 1650, and 1000 cm^{-1}. The bands at 3000-2800 $cm^{-1}$are because of -CH vibrational stretching of alkyl groups,

however the bands at 1382 and 1413 cm^{-1} are produced by -CH2 symmetrical vibrations. The presence of amides in hydrogels is represented by 1653 cm-1 for amide-I, 1560 cm^{-1} for amide-II, and 1322 cm^{-1} for amide-III [21, 56]. The presence of additional peaks may indicate the presence of other components, such as crosslinking agents or other additives. FTIR can also be used to study consequences of various processing parameters on the structure and properties of biopolymer-based hydrogels. For example, changes in the intensity or shape of specific peaks can indicate changes in the degree of crosslinking and the presence of residual solvents [57].

1.7.2. Scanning electron microscopy

The surface structure, topography, and morphology like cracks, pores and roughness surface in hydrogels is characterized by scanning electron microscopy (SEM). The working of SEM is well depicted as it utilizes a fixated ray of light for creating images after scanning of hydrogel sample. Detectors like Everhart-Thornley, silicon drift detectors, and solid-state detectors work efficiently, and notice surface of sample, as a result SEM image is captured. SEM is advantageous as its high-resolution images fortunate researchers by providing them structure morphology, unevenness of texture, and porosity. The achievement of SEM imaging is started just after preparation of hydrogel sample, initially, water or any type of moisture is removed by applying freeze-drying or replacement of it with any other solvent like ethanol. The sample is prohibited from charging and the resulting images should be very much differentiated. Therefore, sample is coated with metal like Au or Pt and undergoes for examination [58].

The SEM analysis for hydrogels is very significant as it estimates the interaction hydrogel with environment and assesses the optimization of hydrogel. SEM technique is applied to do cross-sectional analysis for inspection of internal structure of hydrogels. These evidence might be credited to govern the porosity, interconnectedness, and dispersion of the polymer network within the hydrogels [59]. SEM analysis provides information for alteration in morphology of hydrogels because these have high water capacity or other biological fluids. Owing to this information regarding swelling nature of hydrogel, combined with variations in pore size and physical appearance, explains the mechanisms fundamentals of swelling process [60].

1.7.3. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a popular analytical technique for determining the chemical composition and properties of biopolymer-based hydrogels. This can be accomplished by separating the constituent substances depending on their chemical properties, for example, molecular weight, charge, and polarity and distinguishing them using various detectors, like UV or fluorescence detectors. HPLC is used to measure the degree of crosslinking in hydrogels by determining the volume of nonreactive functional groups in polymeric network. This gives data regarding mechanical and swelling properties of hydrogels. HPLC is used to assess drug release from hydrogels by calculating drug concentrations in the neighboring medium over time [61].

1.7.4. Gas chromatography

Gas chromatography is a method to identify and eliminate sample constituents to assess quantities of individual substances. GC is a valuable technique for characterization of hydrogels because of its ability to detect unknown substances but it usually works under higher temperatures so, it is restricted [62].

1.7.5. Thermogravimetric analysis

Thermogravimetric analysis measures the weight loss of hydrogels as a function of temperature and time. The loss in weight in the samples is the reason of decomposition and oxidation. The

thermal properties of polymeric substances are described as T_g curve. The thermal behavior of biopolymer based hydrogels is analyzed by TGA [60].

1.7.6. Mass spectrometry

The chemical structure and organization of polymer materials in hydrogels can be investigated by MS. MS has the ability to analyze chemical composition and molecular weight of biopolymer-based hydrogels. However, owing to the complex nature of polymers and cross-linking agents, analysis of hydrogels via MS can be challenging and requires new techniques for sample preparation and modern instruments [52].

1.7.7. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy is commonly used in scientific fields, including chemistry and biology. Hydrogels can be studied using NMR spectroscopy. NMR spectra assists scientists to recognize the composition, branching, and molecular weight distribution of polymers. NMR spectra gives information of water molecules in polymer chains [63]. Scientists interrogate how water correlates with the polymer chains and the swelling behavior of the hydrogel. NMR may be used to analyze density of cross-linking in hydrogels, which influences mechanical and swelling properties. The procedures like relaxation time measurements deliver knowledge regarding movement of polymer chains and, subsequently, the cross-linking density [64]. Researchers study variations in polymeric structure and water content for hydrogel swells or de-swells in response to external stimuli by achieving NMR spectra after particular time periods. The diffusion of molecules is investigated inside the hydrogel medium by NMR. This is particularly appreciated for knowing about transport competences of hydrogels, which is important in applications like medication delivery [65]. This is useful in applications like drug delivery, where the volume of the hydrogel to encapsulate and release molecules is vital.

When dilute samples or low-concentrated polymers are used for formulation of hydrogels, NMR spectroscopy can be less sensitive. This can be difficult when examining specific interactions or components present in trace levels. When dealing with overlapping signals, the resolution of NMR might be inadequate for extensive study of complicated hydrogel structures. High molecular weight polymers or complicated network topologies may present spectral resolution problems. The quality of NMR data can be impacted by inhomogeneity within hydrogel samples. Variations in sample composition, local concentrations, or cross-linking density heterogeneity might result in wider and less informative NMR signals. Some NMR experiments, especially those employing multidimensional approaches, might take a long time. This may make researching dynamic processes or real-time changes inside hydrogels more difficult. While NMR is a useful tool for solution-state investigation, its imaging capabilities, such as those seen in magnetic resonance imaging (MRI), may be limited for some hydrogel studies, particularly when compared to alternative imaging techniques with better spatial resolution [66].

1.7.8. Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a thermal analytical method, commonly used to analyze thermal characteristics of hydrogels [67]. DSC deals with heat flow linked with the sample as a function of temperature, gains info regarding phase transitions, thermal stability, and other thermodynamic characteristics. The gelation temperature, or the temperature at which a hydrogel changes from a solution form to a gel state, can be studied using DSC. It can also offer information on the melting temperature or the temperature at which the hydrogel transitions from a gel to a liquid or sol state. DSC investigates cross-linking density by observing various changes in heat volume because of hydrogel structure. DSC is used to govern glass transition temperature (Tg) of amorphous hydrogels [68]. DSC may be used to examine the kinetics of hydrogels. DSC is employed by investigators to thoroughly comprehend thermal properties of hydrogels, which is

essential for optimizing their performance in biomaterials, drug delivery, and tissue engineering [69].

1.7.9. Atomic force microscopy

Atomic force microscopy works for high imaging resolution on nanoscales. It is used to study the hydrogel samples in a broader range and applicable in liquids or air. Since, the working of AFM is based on the interaction between sample and scanning tip of instrument, it plays a significant role in biopolymer-based hydrogels prepared for tissue engineering and wound healing because of giving information related to physical parameters. AFM analyzes mechanical characteristics such as adhesion, elasticity and stiffness of hydrogels, however it does not give pure topography of the samples [70].

1.8. Conclusions

Biopolymer-based hydrogels are more biocompatible, biodegradable, and of natural origin than synthetic hydrogels. Current innovations in biopolymer-based hydrogel synthesis and chemical analysis have made the use of hydrogels in biomedical applications. UV spectroscopy may be used to regulate crosslinking and interactions among biopolymer constituents. The morphology, size, and topology of the hydrogel structure may be determined using SEM. HPLC determines chemical composition, crosslinking, and drug release kinetics. TGA elucidates data on thermal stability, breakdown behavior, and crosslinking degree. A combination of these characterization methods provides a complete package of information about the properties and behavior of biopolymer-based hydrogels, permitting the design and optimization of hydrogels with various applications. Biopolymer-based hydrogels have received fame because of their unique structure, characteristics, and prospective applications in areas such as medication delivery, tissue engineering, and wound healing.

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