REVIEW

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Self-healing, injectable chitosan-based hydrogels: structure, properties and biological applications

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Conventional biomaterials suffer from mechanical stresses and biochemical degradation, compromising performance and structural integrity. Hydrogels are three-dimensional networks that hold a huge quantity of water but can retain their internal structure due to chemical and physical crosslinking. Unlike self-healing injectable hydrogels, they lack the capacity to repair their inherent structure after damage due to the absence of reversible bonds, but these self-healing injectable hydrogels can withstand and reverse damage accumulation. The recoverability of self-healing injectable hydrogels stems from the presence of reversible chemical bonds within their structure. Schiff base linking involves reversible imine or hydrazone bond formation through reactions between aldehydes or ketones, commonly used in chitosan-based hydrogels. Rheological and morphological characterization help determine the precise structure, mechanical strength and bond types. Moreover, the capacity to hold water in hydrogels can closely mimic the extracellular matrix (ECM). Incorporating various polymers and crosslinkers enhances mechanical strength and biocompatibility. Their porous and hydrophilic nature allows for versatile applications, such as loading living cells, drugs, growth factors, and miRNA, promoting cell proliferation and adhesion. The injectability of these hydrogels facilitates precise administration through narrow syringes, enabling rapid local confinement and reducing off-target side effects. Through 3D bioprinting, the injectability can be proven and through experimental studies conducted *in vitro* using dissolution tests or *in vivo* on rodents, the sustained drug release capabilities of these hydrogels can be determined. Chitosan-based self-healing injectable hydrogels possess remarkable properties that find applications in tissue-engineered scaffolds, drug delivery, wound dressings, and cancer treatment.

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1. Introduction

Conventional biomaterials like metal, ceramics, polymers and composites possessing higher mechanical strength experience constant mechanical stresses and undergo biochemical degradation within the biological environment. This can compromise their structural integrity and functional performance. Also, they lose the capacity to undergo structural restoration following damage. Hydrogels, being polymers and soft condensed matter, can be intentionally designed to mimic physiological conditions, but the absence of reversible bonds refrains them from recoverability.

Self-healing injectable hydrogels reveal the unique capacity to withstand and reverse the damage that accumulates over time and external factors. The bio-inspired self-healing process is familiar as many parts of our body self-heal on damage: wound healing,[1] bone re-modelling,[2] liver regeneration,[3] neuronal regeneration,[4] DNA repair.[5,6] In self-healing injectable hydrogels, recoverability arises from the presence of reversible chemical bonds within the hydrogel's structure, allowing it to recover its structural integrity and mechanical properties upon deformation.

Polysaccharides, proteins, and synthetic polymers offer tunable properties to form hydrogels. Chitosan, a polysaccharide is a biopolymer derived from crustaceans, proven to be biocompatible, non-toxic, biodegradable and additionally show the self-healing property on modifying its chemical nature by introducing reversible bonds. Different chemical strategies can be equipped for reversible bonding; however, Schiff base linking is the most commonly used method in chitosan-based self-healing injectable hydrogels, a reaction between aldehydes or ketones to form reversible imine or hydrazone bonds. External conditions such as temperature,[7,8] pH,[9,10] and thixotropy can trigger the self-healing response and gelling strength in hydrogels.

By incorporating various polymers and corresponding cross-linkers, chitosan having free hydroxyl and amine groups can be engineered to closely resemble the physiological environment in addition to enhanced strength and biocompatibility. Loading living cells, drugs, pro-angiogenic cytokines, growth factors, and miRNA offers versatile applications for cell proliferation and cell adhesion because of its porous and hydrophilic nature. The injectability of these hydrogels can be determined by 3D bioprinting, which transforms the given input CAD file into a printed structure. The concept of additional manufacturing paves the way to higher and sustained drug release, and injectability reduces off-target side effects.[11] The minimally invasive administration of self-healing injectable hydrogels through a narrow syringe allows for rapid local confinement of the hydrogel and any incorporated substances.

This review focuses on understanding, designing, and characterizing the inherent behavior of self-healing and injectability in chitosan hydrogels. Chitosan, being insoluble in aqueous phase at neutral conditions, requires structural modifications for its usage in further applications. In addition to structural modifications, derivatives of chitosan often enhance the cross-linking ability and bond reversibility with various other biopolymers. To determine the self-healing and injectability, understanding the structural modification and rheological characterization is significant. Self-healing injectable hydrogel can be locally administered and in desired shapes using 3D bio-printing of hydrogel. Other various experimental tests include characterizing its morphology, and biocompatibility. At a preliminary stage, *in vitro* tests are conducted to observe cell proliferation, cell adhesion in the self-healing hydrogel matrix using various cell lines in 3D bio-printed hydrogel too. Chitosan based self-healing hydrogels also have the potential to work as drug carriers. The presence of a free amine group imparts an inherent antibacterial property to chitosan.[12] Its remarkable properties find application in tissue-engineered scaffolds,[13] wound dressings, drug delivery carriers, and cancer treatment. In wound dressing, these hydrogels can effectively conform to irregularly shaped wounds,[14] providing an ideal solution through a narrow syringe delivery. All the above-discussed applications will be explained in detail concerning hydrogels and, more prominently, chitosan-based self-healing injectable hydrogels (Fig. 1).

2. Self-healing injectable hydrogels

The definition of self-healing has been evolving with technological advancement since the 1990s. Zeroth generation self-healing materials are defined as those that retard, but do not repair mechanical damage. First-generation self-healing materials are defined as those that irreversibly repair but do not restore the damaged matrix, while second-generation materials are based on reversible shape restoration.[15] They can go through numerous cycles of stretching and breaking, but they ultimately mend by themselves. As a result of the hydrogel's inevitable breakdown,[16] cell would diffuse into the surrounding
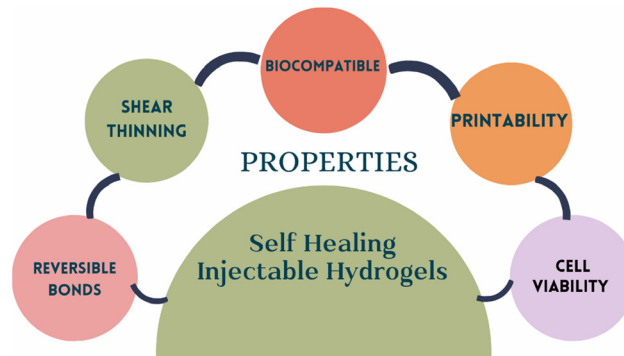

Fig. 1 A mind map representing different properties of self-healing injectable hydrogels, which aid in the potential to be the best alternative for traditional biomaterials. The reversible bonds and shear-thinning nature are the essential properties determining self-healing injectable hydrogels' nature.

tissues and eventually die in the severe ischemia environment. Damage developed during this can be repaired automatically thanks to the self-healing capability. Additionally, self-healing hydrogels having the potential to integrate as bulk gels at the target site can be administered without gel fragmentation.[11,15,17] Sometimes, these self-healing biomaterials need an external trigger like pH,[10,18,19] temperature,[8,20,21] UV or chemical healing agents to self-heal. For such cases, chemical healing agents are generally filled in capsules or tubes and released upon breakage of the element.[11] However, these self-healing injectable hydrogels lack mechanical strength and biocompatibility issues, which can be addressed by employing stronger interactions between biodegradable substances.

2.1 Design & synthesis

Understanding the design strategies for biomaterials is crucial because self-healing originates at the structural level.[17] They are based upon a trade-off between physical and chemical bonding.

The primary design criteria being reversibility and shear thinning facilitate injectability and intrinsic self-healing.[11] non-covalent interactions and dynamic covalent interactions are the different chemical bonding strategies. Non-covalent interactions are over a wide range in nature. Due to their adaptability, they can be used in an aqueous environment. Electrostatic interactions: internal attraction forces between cationic and anionic polymers, metal coordination:[22] *via* chelation, which involves several ligands sequestering metallic ions, hydrophobic interactions, and hydrogen bonding are the different strategies of non-covalent interactions. Dynamic covalent interactions are the chemical crosslinking of polymers by covalent bonds. This is also the traditional approach for hydrogel fabrication. Click chemistry reactions[11,23,24] like Diels–Alder reactions, Schiff-base reactions, and Thiol-Disulfide exchange reactions can be used to design the self-healing injectable hydrogels (Table 1). Diels–Alder reactions involve the [4+2] cyclo-addition of compounds to form a thermally reversible six-membered cycle.[25] These disulphide bonds

Table 1 Synthesis strategies of chitosan-based hydrogels

Polymers	Crosslinking strategies	Ligands or crosslinkers	Biocompatibility tested against	Applications	Brief findings	Ref.
Chitosan–collagen	Schiff base linkages (imine)	DA-PEG	Sprague Dawley (SD) mice model	Strain sensitive epidermal sensor, wound healing	Hydrogel has excellent hemostatic ability, multifunctional based on naturally occurring biomass	19
Chitosan and pectin	Diels–Alder Reaction	Dil. Aq HCl pH = 5	Fibroblast L929 cells	Drug delivery	Swelling effects were observed in Water and PBS, pH responsive Fluorouracil release	27
CMCS-OxHPC	Schiff base linkages	Ketone bonds are introduced	—	Targeted Drug Release	Selective oxidation of secondary OH groups that terminate substituents of HPC to ketones	28
N,O-CMC and OCS	Schiff base linkages	—	NIH/3T3, HUVECs cells	Wound Dressing	The degree of crosslinking, gelation periods, and rheological characteristics of the hydrogel were influenced by varying the mass ratio of N,O-CMC to OCS. Effective reduction in blood loss after surgery	29
Chitosan–dextran	Schiff base linkages	Gallic acid	L929 mouse fibroblast cells, ICR mice model	Combined radiation & burn injury, diabetic foot ulcer	Multiple ROS scavenging ability, humid and antibacterial environment, reduction in inflammatory response on the topical wound site.	30
OHA-HTCCMA	Dual crosslinking; Schiff base, Photo irradiation	LAP photoinitiator	Rabbit articular chondrocytes (CP002), primary bone mesenchymal stem cells (BMSC), New Zealand White rabbits	Osteoarthritis, cartilage regeneration	The hydrogel promoted cartilage regeneration and prevented cartilage lesions in *in vivo* systems	31
CA-pDA	Schiff base linkages	L-Arg, pDA-NPs, CHO-PEG-CHO	HSFs, HUVECs, Mouse RAW 264.7 macrophages SD rat model	Large skin wound repair	Hydrogel can effectively heal large skin wounds, increasing angiogenesis and minimising scarring.	32
SCS	Schiff base linkages	GAMA	Breast cancer cell line (MCF-7), Human embryonic kidney cell line (HEK-293)	Anticancer drug delivery	The hydrogel showed higher pH responsive drug (N-Cur) release at acidic pH. Drug loaded hydrogels inhibited the growth of MCF-7	33
OHAH-CMCS	Schiff base linkages	Hydroxyapatite nanoparticles	L929 cells	Bone repair and regeneration	HA particles showed strong interface compatibility with the organic matrices, making it a viable material for the application of bone regeneration and repair.	34
Chitosan–cellulose	Schiff base linkages	Cellulose nanofibres	Neural stem cells	Neural regeneration	Hydrogel showed excellent space remodeling effect and nutrient transportation	35

Abbreviations: DA-PEG – dibenzaldehyde-modified PEG2000; PF – Furan modified Pectin; maleimide-modified chitosan (CA); CMCS – carboxymethyl chitosan; Ox-HPC – oxidized hydroxypropyl cellulose; N,O-CMC – N,O-carboxymethyl chitosan and OCS – oxidized chondroitin sulfate; OHA – oxidized hyaluronic acid; HTCC N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride; HTCCMA – N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride methacrylate; LAP – lithium phenyl-2,4,6-trimethylbenzoylphosphinate; CA – L-arginine conjugated chitosan; CHO-PEG-CHO – benzaldehyde group functionalized poly ethylene glycol pDA-NPs – polydopamine nanoparticles; CA-pDA, as an angiogenic and antibacterial dressing for wound repair. GAMA – gum arabic with multialdehyde groups; SCS – succinic anhydride-modified chitosan; N-Cur – nanocurcumin; OHAH – oxidized hydroxyapatite/alginate hybrids.

Fig. 2 The chemical strategies for designing self-healing injectable hydrogels, including non-covalent and dynamic covalent bonds. Figure reproduced from Bertsch *et al.* 2022[11] open access article under Creative Commons Attribution License with CC-BY-NC-ND.

are multi-responsive to pH, light and redox reactions.[26] In Schiff-base linkages, the nucleophilic group attack of amines on the aldehydes and ketone groups creates reversible bonds (Fig. 2).

To address the disadvantages of covalent and non-covalent interactions, a new class of introductions were introduced, which includes both covalent and non-covalent interactions. Dual cross-linked hydrogels and double network hydrogels come under this class. Non-covalent interactions are weak and sensitive to specific external triggers like pH or ionic strength. In combination, excellent results were observed where the presence of imine bonds in Schiff base reactions combined with other non-covalent interactions.

The hydrogel network's physical configuration and spatial topology substantially influences the resulting hydrogel system's external and internal characteristics. Different physical strategies to design a hydrogel include monolithic hydrogels, Fibrous hydrogels, colloidal and granular hydrogels, particle cross-linked hydrogels, and particle-filled hydrogels. As the name suggests, mono means one, *i.e.*, hydrogel is made using one polymer. The nature of fibrous hydrogels is to impact mechanical and biological properties. Ruquan Zhang[36] *et al.* said Xanthan Gum is a weak hydrogel because of the presence of hydroxyl and carboxyl groups. But on fabricating the same

with a fibrous protein like silk fibroin,[36] krill protein composite fiber[37] the mechanical strength increased substantially. In a similar fashion, to address the issue of biocompatibility, biopolymers like silk fibroin, chitosan, dextran, alginate, cellulose, starch, pullulan, and hyaluronic acid can be used to make self-healing injectable hydrogels improving both biocompatibility and mechanical properties by introducing fibrous, colloidal/granular particulates (Fig. 3).[11,38]

2.1.1 Structure and design strategy of chitosan. Chitosan is a natural cationic polymer derived from the *N*-deacetylation of chitin. Chitin can be obtained from fungal *mycelia*, crab shells, or shrimp shells.[39] The other method of production of chitosan is by fermentation processes, which includes alkali treatment to yield chitosan-glucan complexes. Chitosan is composed of *N*-acetyl glucosamine and glucosamine units. Unlike other polysaccharides, chitosan is highly basic and has been widely used in pharmaceutical and surgical procedures for ophthalmology, wound dressing, tissue regeneration, cancer-treatment, *etc.* because of its biocompatibility, biodegradability and antimicrobial property. Other than biomedical applications, it can be used in ref. 39. Photography and packaging[40] (films), cosmetics,[41,42] artificial skin,[43] food & nutrition,[44,45] application in solid-state batteries,[46–48] and to impart wet strength to paper.[49]
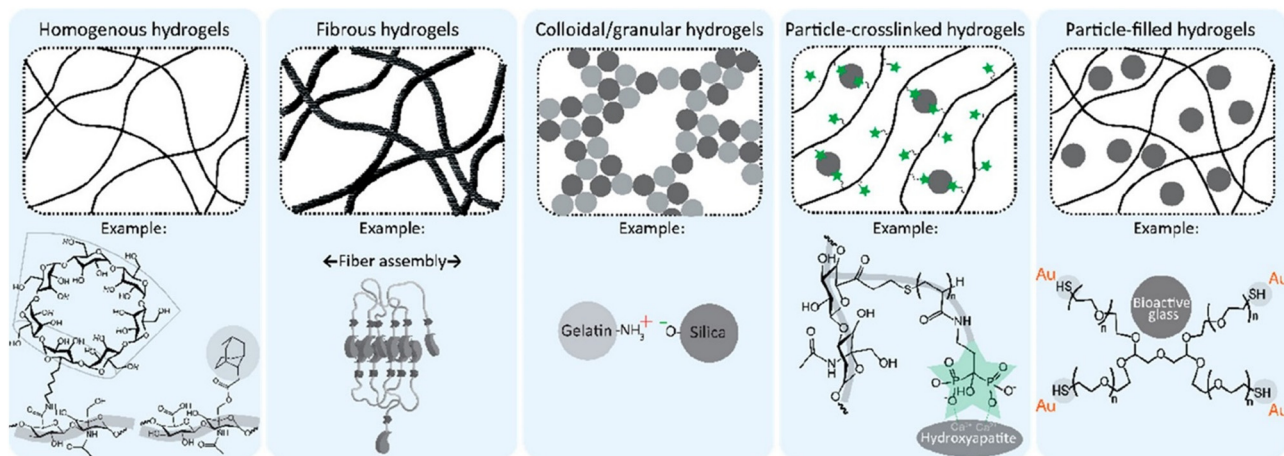
Fig. 3 The overview of physical design strategies of self-healing injectable hydrogels. Monolithic/homogenous hydrogels: impact the network topology of resulting hydrogels; fibrous hydrogels: mechanical stability, colloidal hydrogels: cell ingrowth; particle-crosslinked: mechanical properties and self-healing, particle-filled hydrogels: particles act as fillers to reinforce hydrogels reproduced from Bertsch *et al.* 2022 ref. 11 open access article under Creative Commons Attribution License with CC-BY-NC-ND.

The nucleophilic groups in chitosan facilitate Schiff base reactions, forming reversible bonds. In these reactions, the free amine group in chitosan reacts with aldehyde or ketone groups, creating dynamic and covalent imine or hydrazone bonds. For the progress of the Schiff base reaction, a crosslinker with aldehyde groups is required. Xu Y. *et al.*, suggested four different strategies to generate polysaccharides with aldehyde groups.[50] The first strategy is the production of polysaccharides with inbuilt aldehyde through the oxidation of hydroxymethyl groups leading to glycol cleavage with the help of sodium periodate to form aldehyde products. This method of periodate-mediated formation of aldehyde group was utilized in polysaccharides like gum Arabic,[51,52] alginate,[53–55] chondroitin sulfate,[29,56–58] dextran,[30,59–61] hyaluronic acid,[31,62–65] pectin.[66–69] In the second strategy, the polysaccharide is generated so that aldehyde groups are present on the ends of the linear polymers as the crosslinking agent.[50,70] Small dialdehyde molecules serve as the best crosslinking agents for this strategy, but their toxicity limits its application.[20,70] One of the few biocompatible, water-soluble, and non-toxic polymers is polyethylene glycol or PEG. Hence, dialdehyde derivatives of PEG have been created to address this toxicity issue. Dibenzaldehyde functionalized polyethylene glycol is the widely used crosslinker to form aldehyde groups at the ends of linear polymers.[32,70–72] The above crosslinker is formed by the esterification of hydroxyl-terminated poly ethyl glycol with 4-formyl benzoic acid (FBA).[33,73,74] The other method strategizes crosslinking with star polymers consisting of an aldehyde group at the end of each arm of the polymer, forming a star-like PEG.[75] Multi-aldehyde PEG, multi-armed PEG crosslinkers,[76] and star-shaped eight-armed PEG crosslinkers[77] create possible ways for Schiff base reactions to increase the crosslinking sites, thereby increasing the gelation time, biodegradability, and self-healing ability. This strategy can be regarded as the appropriate choice to play with as it can create strong self-healing hydrogels.[76–78] reveal remarkable self-healing abilities visualized macroscopically and rheologically on the addition of polyethylene glycol(PEG).

The third method utilizes linear polymers as crosslinking agents with many side chains containing aldehyde groups. One main advantage over the previous strategies is that the variation in the concentration of the crosslinker increases both mechanical strength and rate of biodegradation. Chitosan can be blended with other polysaccharides of the same or different family depending on the degree of miscibility and number of polymer chains as another strategy. Alginate,[34,55] cellulose,[35,38,67,79,80] and gelatin,[55] with the help of chemical oxidation, convert the hydroxy groups into aldehyde bonds. This combination of blending of various polysaccharides with chitosan enhances the functional, mechanical, and self-healing ability of chitosan (Fig. 4).

Due to its strong intermolecular hydrogen bonding, chitosan is insoluble and unstable in neutral and high pH solutions, necessitating the use of acids to prepare chitosan solutions. Typically, either citric acid or acetic acid is employed for this purpose; however, it is necessary to perform continuous washing to eliminate any excess acid. The presence of free amine on C_2 and free hydroxyl groups on C_6[81] in chitosan is exploited to synthesize its derivatives by methods like methylation, carboxylation, quaternization, sulphation, and phosphorylation,[82] making the process much simpler than using acids. The hydroxyl group present on C_3 in Fig. 5 has high stearic hindrance, so it cannot react,[83] and hence only functional groups of C_2 and C_6 are readily reactive A clear depiction of the chitosan structure is given in the figure below. The basic idea behind synthesizing its derivatives is to weaken the intermolecular hydrogen bond, making it water-soluble. These derivatives are not only soluble in water but over the whole pH range. They could retain the biological properties of chitosan by improving the chemical and physical properties for various biomedical applications (Fig. 5).
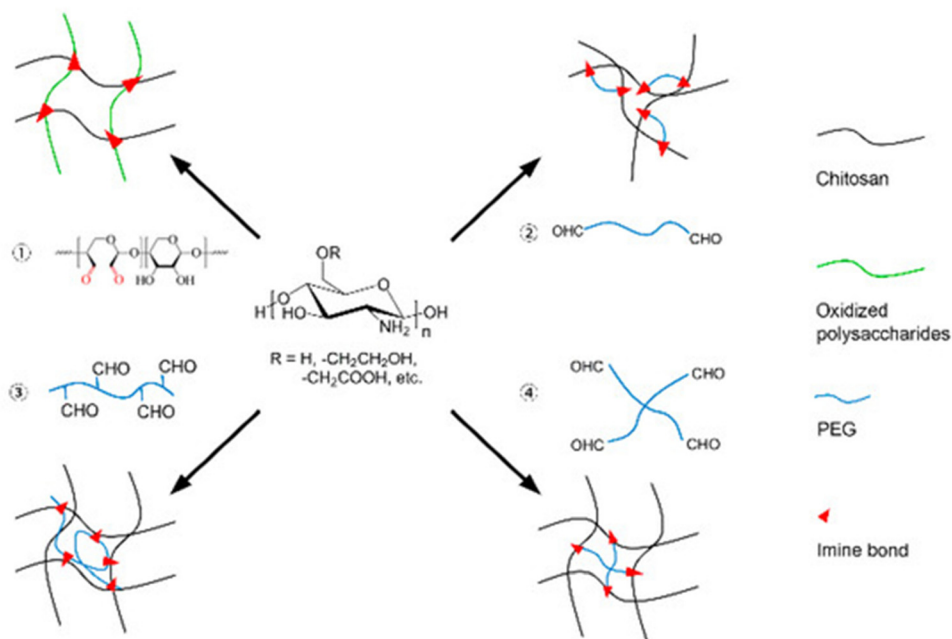
Fig. 4 Representation of four different strategies to introduce aldehyde functional groups into chitosan, enabling the formation of Schiff base linkages. PEG abbreviates to poly-ethylene-glycol Reproduced from ref. 50 (Xu *et al.*, 2018) For articles published under an open-access Creative Common CC BY license.
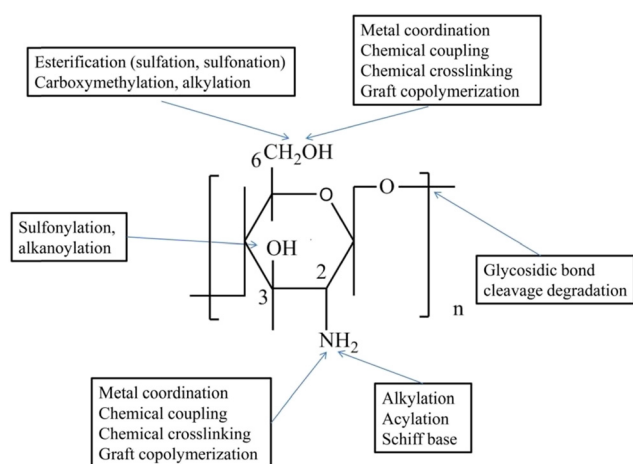

Fig. 5 The processes to synthesize derivatives of chitosan. The hydroxyl groups present on C6 and C3 Reproduced from ref. 83 Wenqian Wang *et al.* 2020 open-access articles distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

2.1.2 Chitosan derivatives

Chitosan oligosaccharides (COS). Produced *via* enzymatic or acid hydrolysis of chitosan, they represent truncated chains of the original polysaccharide. This diminutive structure has low molecular weight and shorter chain lengths, readily water soluble, thereby opening vistas to explore its health-promoting attributes, encompassing antioxidant, antitumor, and antimicrobial properties with biodegradation in kidney tissues, plasma, hepatic cells, and urine by lysosomes.[84] Its higher water solubility allows for easy absorption of COS in intestinal epithelia thereby blood hence permittable to whole body. COS is proven to show excellent results in wound healing in moist environment due to water solubility.[85] The presence of many free amine groups makes it compatible for further structural modifications with both biopolymers and synthetic polymers. Although COS has potential therapeutic applications for various diseases, it is proven that at higher concentrations (70 μg mL^{-1}), COS causes cell death in human lymphocytes. Inadequate animal studies cause a lack of safety and impurity profiles data of COS. It is imperative to recommend thorough safety and chemical investigation when COS is exposed to chemical, enzymatic, or thermal exposure.[86–88]

Chitosan hydrochloride. Through a chemical reaction involving chitosan and hydrochloric acid, the formation of chitosan hydrochloride transpires. The ensuing product chitosan hydrochloride, a chitosan derivative salt, evinces heightened water solubility compared to native chitosan, rendering it amenable to application within diverse biomedical contexts.[81] Chitosan hydrochloride has mucoadhesive properties, meaning it can adhere to mucosal surfaces like gastrointestinal (GI) tract and respiratory tract where important functions like absorption and excretion occur. Mucoadhesive property is advantageous in drug delivery or in systems requiring prolonged contact with biological tissues.[89] Permeability of chitosan hydrochloride is much higher in intestinal epithelial cells when compared to chitosan.[90] In addition to biomedical applications, chitosan hydrochloride can be equipped in applications of air filtration membranes,[91] coating,[92,93] baking,[94,95] plant immunity inducer.[96,97] This derivative of chitosan is also used as a hydrogel coating in wound dressings exhibited efficient

antimicrobial properties.[98] At the same concentration levels chitosan hydrochloride has poor antioxidant activity when compared to Chitosan oligosaccharides. Also, its antimicrobial activity varied with the type of target microorganism which was proven by ref. 99.

Carboxymethyl chitosan. Leveraging the introduction of carboxymethyl groups onto chitosan molecules, carboxymethyl chitosan emerges as a consequence. The replacement of free hydroxyl and amino groups with carboxyl functional groups confers augmented water solubility to chitosan, thus engendering its appropriateness in a plethora of therapeutic and biomedical pursuits, including drug delivery systems and wound healing therapies.[29,53,71,74] In addition to Schiff base bonding, the presence of abundant free carboxyl and amino group can confer to form coordination bonds with metal ions like $Fe^{(3+)}$,[100,101] $Al^{(3+)}$,[101,102] $Ag^{(+)}$.[103,104] Carboxymethyl Chitosan has improved antibacterial and antioxidant properties due to the intermolecular non-covalent interactions. It shows antimicrobial activity against *E.coli, s.aureus, Candida tropicalis, candida parapsilosis, candida krusei, candida glabrata, B.subtillis.*[105] The higher number of chelating groups improves the metal ion sorption properties of chitosan. Its moisture retention properties is advantageous for better wound healing, cell adhesion and cell proliferation.

Quaternized chitosan. It is brought to fruition by grafting quaternary ammonium groups onto the chitosan backbone. Different ways of grafting quaternary ammonium groups include Schiff base reactions,[106] addition of glycidyl trimethylammonium chloride (GTMAC)[107,108] in hydrophilic chitosan, etherification in alkaline condition, direct dissolution of chitosan in alkaline solvents like LiOH/KOH/urea-aqueous condition at low temperatures.[109] Although studies reveal that grafting quaternary ammonium group onto chitosan is tedious and time taking[109,110] its chemical modification imparts a heightened propensity for water solubility and accentuates the antimicrobial properties of chitosan. Consequently, quaternized chitosan attains utility in an array of applications, such as antimicrobial agents, drug delivery vehicles, and wound dressings.[59,66] Similar to chitosan hydrochloride, quaternized chitosan shows enhanced mucoadhesive properties due to increased solubility and cationic character therefore, it can strongly interact with anionic groups of mucin, thereby binding with the epithelial surfaces[111] *N,N,N* trimethyl chitosan (TMC) is the simplest form of quaternized chitosan. Increasing the degree of quaternized groups increases the antioxidant properties.[112] In fact, increasing the concentration of Quaternized chitosan tuned and improved the mechanical properties of self-healing injectable hydrogels.[107,113,114] Quaternary chitosan revealed applications as a strain sensor effectively adhering to different substrates and contributed efficiently towards electrical conductivity and electrical sensitivity when ions like Fe^{3+} and Cl^- are added. Hence showing applications in human health and motion detection[110]

Carbamoylated chitosan. Carbamoyl group ($CONH_2$) is induced onto the chitosan molecule. Various reactions equip carbamoylating agents like isocyanates or carbamoyl chlorides. In addition to enhancing the water solubility and mechanical properties, carbamoylated chitosan serves as the best carrier for drug delivery and cell growth applications due to its non-cytotoxicity[115,116] Studies are needed to determine the self-healing nature of this biocompatible injectable chitosan hydrogel. Its applications involve in tissue engineering and 3D printing.

3. Characterization and properties of chitosan-based hydrogels

3.1 Rheological properties

3.1.1 Recoverability. The synonym of self-healing is regaining its original structure from the damage caused by mechanical and biological stresses. During rheological measurements, various properties like Shear stress, extent of deformation, temperature, and the normal force exerted by the sample influence the viscoelastic properties. The presence of reversible bonds in modified chitosan hydrogel induces a self-healing ability. Recoverability can be determined in two ways. The first one includes cutting the hydrogel into two separate discs (as in Fig. 6(A) and (G)). After a while, these cut pieces join back into their original shape without any visual cracks.[117,118] For a much clearer depiction, often researchers dye each part of the hydrogel into a different color using rhodamine[52,119] and sometimes methylene blue[33,120–122] dyes in general. The second method of determining recoverability involves step-strain measurements using oscillatory or rotational measurements in a rheometer. During the measurement process, the self-healing injectable hydrogel undergoes simultaneous deformation and healing while varying the shear or strain rates applied to it in cycles. The extremities of the extent of deformation are defined by using the strain sweep measurements in a rheometer, which measures the point of yield stress, where the storage modulus (G') and loss modulus (G'') meet (precisely, a point where their tangents meet to avoid over-estimation) at a constant frequency of 1 Hz (angular frequency of 10 rad s^{-1})[123,124] or, 6.28 rad s^{-1} or, 0.1 Hz,[125] a point where the hydrogel breaks and from there on the hydrogel no longer can be retrieved back in any means to its original gel state but remains in liquid phase. The step-strain test, also known as alternate-strain or 3iTT (3 interval-time-thixotropy test), is time-dependent and controlled at varied shears beginning with a lower rate. Fig. 6(D) shows the rheological measurement in 3 cycles with varying at 1% strain rate and 500% strain rate.

3.1.2 Injectability. The second word in the title chitosan based self-healing injectable hydrogels, injectability, is an important criterion to be considered. For any hydrogel to be injectable, it has to be a shear-thinning fluid. According to the power law, all thixotropic fluids are non-Newtonian fluids whose flow behaviour index (n) <1 and shear decreases with

time. These fluids reveal injectable properties. The basic idea behind self-healing injectable hydrogels is that fluids resist the shear on extrusion and solidify to gels after extrusion. Flow curve measurements aid in determining the viscosity behavior with varying shear rate. A gradual decrease in viscosity on increasing the shear rate depicts a shear thinning fluid *i.e.* its injectability. A visual test can also be performed by loading the liquid hydrogel into a syringe. Hence injectability and self-healing analysis are two main rheological tests for self-healing injectable hydrogels. Major applications of injectable hydrogels include 3D printing of hydrogels for a minimally invasive local administration.[128]

3.1.3 Mechanical strength. Although hydrogels have a huge ability to mimic the extracellular matrix, their mechanical strength is a hindrance. Rheological Measurements, tensile strength, and compressive strength measurements aid in determining their mechanical strength. Frequency sweeps in a rheometer apply an amplitude oscillatory deformation in the linear viscoelastic region over a range of frequencies. This test allows us to determine the equilibrium gel strength, more specifically the storage and loss modulus. In Fig. 6(F), Anda Mihaela Craciun *et al.* (2022) tested how the G' and G'' varied on several dilutions. The modulus values decreased, and the gelation time increased from instantaneous to 30 min on
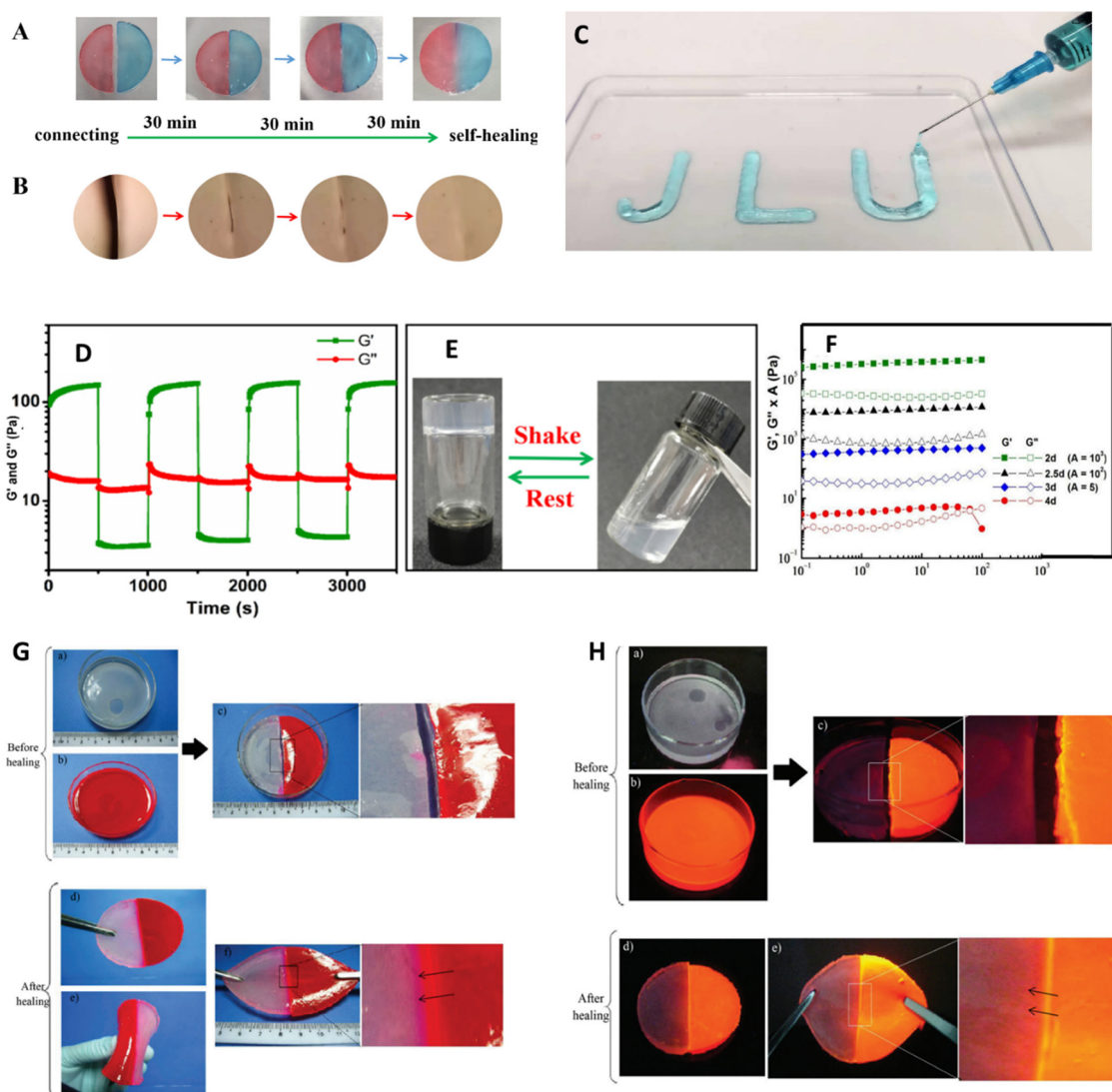

Fig. 6 The rheological properties (A) of the two halves of the gel loaded with methyl orange and methylene blue to determine the self-healing properties after 1.5 h contact time. (B) Microscopic image of (A) revealing its structural recoverability. (C) Injectability of the hydrogel. (D) 3iTT test was performed at 500% and 1% strain rates. (E) inverted test tube test to observe the gelation time physically (F) depicts the curve obtained from the frequency sweep of chitosan and pyridoxal 5-phosphate (P5P) hydrogel loaded with vitamin B6 on diluting at different dilutions. The order of water content is as follows 2d < 2,5d < 3d < 4d; (G) and (H) depicts the visuals of bending and tensile strength of the recovered sample after healing with and without UV. The above images are reproduced from Copyright @ 2023 et al., Yongyan Yang,[126] Anda Mihaela Craciun et al. (2022)[127] open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (**https://creativecommons.org/licenses/by/4.0/**). and Sirajuddin et al. (2014)[119] licensed under creative commons (CC BY-NC-ND 3.0).

Fig. 7 The porosity is increased with increasing the water quantity in the hydrogel composition from 97.88% to 99.30% reported by Craciun *et al.* (2022) (A)–(F).[127] The variation of pore size is increased with the degree of substitution (DS) reported by Fengjiao Zhang *et al.* (2023)[136] (G)–(J). Figures are reproduced from open access articles.

increasing the water content.[119,127] The healed hydrogels are also proven strong enough, similar to the undeformed hydrogels on applying tensile and bending forces, according to Fig. 6(G) and (H).

Rheological measurements determine the flow behavior and mechanical strength at oscillatory and rotational stresses. To determine its mechanical strength under tensile and compressive stresses a UTM can be equipped with sample dimensions according to the ASTM standards.[129]

3.1.4 Pore size & morphology. Porosity influences the performance and efficacy of chitosan based self-healing injectable hydrogels. It refers to the presence of void spaces or pores within the material, the larger the pore size, the higher the porosity. In self-healing injectable hydrogels, porosity facilitates the swelling, infiltration of cells, and the exchange of nutrients, oxygen, and waste products.[130,131] Increased pore size enhances external pH-stimulated self-healing behavior in chitosan hydrogels.[78,127] The channels generated in the hydrogel allow host cells migration and proliferation into the damaged tissue[132] and eventually swap out the malfunctioned organs. Cell adhesion and cell proliferation rate can be improved.[133] High porosity provides a large surface area for

drug loading, and the interconnected pores enable the controlled release of therapeutic agents. A suitable porosity influences the overall mechanical strength, elasticity, and stability of the hydrogels.[79] Morphology refers to the structure, shape, and arrangement of the hydrogel components. The morphology of self-healing injectable hydrogels influences the structural stability of hydrogel and prevents premature degradation or disintegration of the hydrogel upon injection or during the healing process; it also impacts its interaction with surrounding tissues or substrates. By tailoring the morphology, it is possible to optimize adhesion, integration, and interfacial properties, allowing for better tissue integration and enhanced performance of the hydrogel. Morphology and porosity characterization are done using a scanning electron microscope. The pore diameter of hydrogels can be measured using software like ImageJ. The samples of self-healing injectable hydrogels are freeze-dried and sputter coated with gold[126,134] or platinum[59] or chromium. In most cases, the pore size decreased with the increase in concentration of the substrate. At the same concentration, the pore size increased by increasing the degree of substitution (DS) (Fig. 7G–J).[135] Additionally, Water

content present in the chitosan matrix defines and varies the pore size effectively.[127]

3.1.5 Swelling property. Freeze-dried samples are weighed and soaked in PBS or DI water inside a vial. After a certain period, the self-healing injectable hydrogel attains an equilibrium that depicts the maximum absorption capacity of the hydrogel. The excess PBS[137] or DI water[59] is removed with a pipette[137] or filter paper,[59] and the swollen sample is weighed again. This swelling property is directly related to the porosity discussed above. Higher void space creates pockets for the absorption of PBS or DI Water. The equilibrium swelling ratio can be calculated by the ratio of the weight of media present in the sample to the weight of the dry sample.[137] Swelling can support cell proliferation, influences, encapsulation and stiffness. Also, it should be noted that the aqueous media can be changed based on the application of the hydrogel.[74] The degree of hydrogel swelling affects the drug release behaviour by regulating the pace at which the penetrant diffuses into the hydrogel matrix as wells as the drug's dissolution and diffusion throughout the swollen hydrogel matrix. Higher polymer concentrations can therefore produce hydrogels with longer-lasting medication release.[38]

$$\text{Swelling ratio} = \frac{W_s - W_d}{W_d}$$

$$\%\text{Swelling rate} = \frac{W_s - W_d}{W_d} \times 100$$

W_s = weight of swollen sample W_d = Weight of dried sample.

3.1.6 Water retention properties. Naturally formed hydrogels have much higher water absorption and retention properties when compared to synthetic hydrogels.[126] This moisture retention creates an environment conducive to the self-healing process, facilitating the reformation of bonds and structural integrity upon damage. Adequate moisture content helps maintain the structural integrity of the hydrogel matrix and increases the longevity during application. Optimum moisture levels within the hydrogel ensures a suitable environment for cellular activities, wound dressing,[129] flame retardants tissue regeneration. Drug delivery systems utilizing chitosan-based hydrogels moisture retention influences the dissolution, rate and pattern of drug release. Proper moisture levels can help control the diffusion or release of therapeutic agents, ensuring optimal dosage and therapeutic efficacy. Pore size, swelling ratio and crosslinking density influence the rate of drug release. Studies show a sustained drug release for higher crosslinking densities as the pore size is decreased and thereby the water absorption and retention.[38] Moisture content also affects the long-term stability of hydrogels. Optimizing moisture retention mechanisms, such as cross-linking density or incorporating moisture-retaining additives like glycerol, can enhance stability. Water retention ratio is the percentage of water present in the hydrogel after a certain period of immersion per initial water content in the hydrogel.[138] Mathematically, eqn (1) depicts the formula for quantification, W_t denotes the weight of the hydrogel after swelling or deswelling, W_d is the

weight of the dry hydrogel and W_i denotes the weight of the initial hydrogel.

$$\text{Water retention ratio} = \frac{(W_t - W_d)}{(W_i - W_d)} \times 100 \qquad (1)$$

Chitosan owing to its intricate polymeric network has the potential to retain both bound and unbound water molecules. Characterizations like DSC and Raman spectroscopy have been equipped by Mohammad H. Mahaninia *et al.* 2023 to study the role of water molecules present inside the chitosan polymeric network.[139] Jiawei Lu *et al.* 2023 studied the construction of a bilayer chitosan hydrogel possessing a lasting 7 day water retention rate of 64.91%.[129]

3.2 Printing

Additive manufacturing (AM), also known as 3D printing (3DP), involves the sequential addition or deposition of materials, either a single material or multiple materials, to construct a three-dimensional (3D) object layer by layer. Injectability, in the context of 3D printing, is a synonymous term for printability, encompassing several crucial aspects such as extrudability, shape fidelity, and filament classification. For 3D printing to be successful with hydrogels, it necessitates fulfilling these aforementioned factors.[11] Extrudability delineates the hydrogel's flowability during the printing process. At the same time, filament classification elucidates its shear-thinning property, wherein it experiences a phase transition from a liquid state to a gel-like state. Once the hydrogel attains the desired gel structure, it must retain this configuration to prevent filament fusion, denoted as shape fidelity. Shear-thinning hydrogels exhibit rapid gelation properties, whereas stimulus-responsive hydrogels exhibit slower gelation speeds.[140] This extreme gelation kinetics can potentially lead to either clogging of the printing nozzle or the dispersion of printed structures. The former scenario raises concerns about extrudability, whereas the latter compromises the precision of shape fidelity. Thanks to the ingenious implementation of dynamic reversible crosslinking, hydrogels can navigate these challenges. This mechanism enables the hydrogel to flow within the printing extruder under the application of pressure, allowing for smooth extrudability. Subsequently, once the printing process is complete, the hydrogel recovers its structure, ensuring the desired shape fidelity of the printed hydrogel.

3D bioprinting has applications in

(a) Tissue engineering and regenerative medicine in bone, liver, neural, heart valve, cardiac, lung, cartilage, pancreas, and skin

(b) Transplantation and clinics in bone, cartilage, and skin

(c) Pharmaceutics and drug testing

(d) Cancer research.

3D bioprinting is capable of fabricating a diverse range of biomaterials with or without cellular components, utilizing precise and controlled spatial arrangements, ultimately leading to the formation of targeted tissues or organs.[141] Three-dimensional printing, also referred to as rapid prototyping (RP), employs computer-generated data such as

computer-assisted design (CAD), which can be developed using magnetic resonance imaging (MRI) or computer tomography (CT) and transforms them into molded 3D objects. This translation requires a 3D bioprinter which can run by different mechanisms[142] like extrusion-based bioprinting, stereolithography (SLA)-based bioprinting, inkjet bioprinting, and laser-assisting bioprinting. Bioink, a substrate that promotes cell proliferation and cell adhesion, is extruded intricately from the nozzle of the syringe in a pressure-controlled manner onto the printer bed.[143] A 3D bio-printed tissue or organ can either undergo incubation in a bioreactor in a laboratory setting *in vitro* to mature before surgical implantation or can be printed *in situ* using the animal body itself as a bioreactor.[144] But the pressure applied may affect the cell survival rate. Hence as a safer side, cells are seeded after the printing (Fig. 8).

A quick crosslinking approach is crucial to maintaining the structural fidelity of the printed self-healing injectable hydrogel. Crosslinking methods like thermal crosslinking, pH-induced gelation, photo-responsive, and ionic gelation.

3.2.1 Thermal crosslinking. Utilizes temperature changes to induce gelation and create a stable network in chitosan-based hydrogels. This can be achieved by incorporating thermosensitive polymers or additives that undergo a sol–gel transition at specific temperatures.[146,147]

3.2.2 pH-induced gelation. Takes advantage of the pH sensitivity of chitosan to form a hydrogel structure. By adjusting the pH of the chitosan solution, the chitosan molecules undergo conformational changes and interact to form physical crosslinks.[148]

3.2.3 Photo-responsive crosslinking. Involves incorporating light-responsive moieties or photo-initiators into the chitosan-based ink. Upon exposure to specific wavelengths of light,[149–151] these moieties or initiators initiate crosslinking reactions, leading to gelation.

3.2.4 Ionic gelation. Relies on the interaction between chitosan and multivalent ions to form physical crosslinks. By incorporating divalent or trivalent ions, such as calcium or zinc ions, into the chitosan solution, gelation occurs through the ionic interactions between chitosan chains and the ions.[148,152] regarded tripolyphosphate and[153] glutaraldehyde as the best crosslinkers, which impart high mechanical strength and structural integrity. Their combination could reveal better results but showed poor cell viability.

Chitosan hydrogels have emerged as promising biomaterials in 3D bioprinting due to their biocompatibility, biodegradability, non-immunogenicity, low cost and ability to mimic the extracellular matrix.[154] By incorporating bioactive molecules and cells into chitosan-based bioinks, researchers can precisely fabricate complex 3D structures that mimic native tissues, holding significant potential in regenerative medicine for fabricating constructs such as cartilage, skin, and blood vessels.[155] Moreover, the tunable mechanical properties of chitosan hydrogels allow for customization according to specific tissue requirements, further enhancing their utility in 3D bioprinting for creating functional tissue substitutes.

Intini C. *et al.*, designed and fabricated 3D printed chitosan-based scaffolds for the treatment of wounds in diabetic rat model.[156] After 20 and 35 days of incubation, 3D cell cultures displayed significant qualitative and quantitative *in vitro* cell growth, verified by neutral red staining, MTT assays, and scanning electron microphotographs. Combining Nhdf and HaCaT cells and filling scaffold holes after 35 days resulted in optimal cell proliferation on 3D scaffolds, forming an early skin-like layer composed of keratinocyte and fibroblast cells.

Fig. 8 Schematic flow chart of Organ 3D printing (Lazaridou *et al.* 2022)[145] (A), and the progress in 3D printing concerning various applications (Vanaei *et al.* 2021)[142] (B). Open access Creative Common CC BY license (CC BY-NC-ND 4.0).

Table 2 List of chitosan-based hydrogels used for 3D bioprinting

Chitosan	Other polymers/ ligands/materials	Preparation method	Testing of days	In vitro/in vivo models	Disease/targeting applications in	Brief findings	Ref.
Chitosan scaffolds	Acetic acid, raffinose	Extrusion	35 days	Nhdf and HaCaT cells, Wistar rats	Wound healing in diabetes condition	In vitro and in vivo studies suggested 3D printed scaffolds improves the quality of restored tissues	156
Chitosan hydrogel	Gamma-PGA	Double extrusion	14 days	Human adult fibroblasts	Biomedical applications	70% of surviving cells remained in the in the hydrogel with maintained viability over a 14-day incubation period	160
Chitosan	BGP, HEC and CNCs	Extrusion	21 days	MC3T3-E1 cells	Osteogenic differentiation	Cell viability remained uncompromised by the bioinks. CNCs enhanced the osteogenic potential of MC3T3-E1 cells within chitosan scaffolds	157
Chitosan hydrogel	PCL, TFNA	Extrusion	21 days	SMSCs, Rabbits	Articular cartilage (AC) injury	Enhanced cell proliferation, chondrogenesis and promote AC regeneration	159
Chitosan bioink	GE	Extrusion	7 days	KC and HDF cells	Skin regeneration	MTT assay revealed that 93% of the cells in the printed construct were viable	158
Chitosan hydrogel	Gelatin, BGP, sodium bicarbonate	—	—	MSCs	3D models and tissue engineering	The most viable encapsulated mesenchymal stem cells were found in CH2%, and the support bath-assisted bioprinting method	161
Chitosan hydrogel	MA and tricine		7 days	MC3T3-E1 cells	Tissue engineering applications	When encapsulated in CHTMA-Tricine constructions, MC3T3-E1 pre-osteoblast cells are shown to be cytocompatible and to remain viable for six days	162
Chitosan hydrogel	PVA, gelatin and LEV	Physically cross-linking	24 hours	NHF cells	Tissue engineering applications	Cytotoxicity and cytocompatibility tests confirmed the cytocompatible nature of scaffolds	163
Acidified chitosan	RHCMA	Cross-linking	72 hours	HUVECs cells	3D bioprinting and tissue engineering	In vitro studies confirmed biocompatibility of CS-RHCMA bioinks	164
Chitosan	GA	Self-crosslinking	7 days	NIH3T3 cells	Tissue engineering and regenerative medicine	3D-printed CS-GA bioink scaffolds exhibited good cytocompatibility and biodegradability	165

Abbreviations: PGA: polygamma-glutamic acid, BGP: β-glycerophosphate, HEC: hydroxyethyl cellulose, CNCs: cellulose nanocrystals, PCL: poly(ε-caprolactone), TFNA: tetrahedral framework nucleic acid, SMSCs: synovial mesenchymal stem cells, GE: genipin, MSCs: human bone marrow mesenchymal stems cells, PVA: poly(vinyl alcohol), LEV: levofloxacin, RHCMA: recombinant human collagen methacrylic anhydride, HUVECs: human umbilical vein endothelial cells, GA: gallic acid.

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In vivo studies done on streptozotocin-treated diabetic rats also showed enhanced tissue repair quality using 3D printed scaffolds compared to commercial patches and spontaneous healing. In another study conducted by Maturavongsadit P. *et al.*, the use of chitosan and nanocellulose as bioink for 3D printing applications in osteogenic cell differentiation was demonstrated.[157] Glycerophosphate, hydroxyethyl cellulose, thermogelling chitosan, and cellulose nanocrystals (CNCs) constituted the bioinks. Impact of CNCs and pre-osteoblast cells (MC3T3-E1) on scaffold mechanics, bioink printability, and rheology was assessed. CNCs and cells (5 million cells per mL) significantly enhanced bioink viscosity and chitosan scaffold mechanical properties post-production. Cell viability remained uncompromised, with bioinks printable within an optimal pressure range (12–20 kPa). In chitosan scaffolds, the inclusion of CNCs enhanced the osteogenesis of MC3T3-E1 cells. This CNCs-incorporated chitosan hydrogel represents a versatile bioink with appeal for 3D bioprinting, facilitating the production of scaffolds for bone tissue engineering and other medical applications.

Moreover, bioinks integrated with cells were successfully bioprinted, demonstrating better cell viability. Hafezi F. *et al.*, developed chitosan based bioink laden with keratinocytes and human dermal fibroblasts cells.[158] MTT assay revealed that 93% of the cells in the printed construct were alive after crosslinking, processing, and seven days of exposure to physiological conditions. Li P., and team also developed synovial mesenchymal stem cells (SMSCs) contained chitosan and PCL based hydrogels for the cartilage regeneration.[159] This 3D printed chitosan/PCL scaffold provide favourable microenvironment for the proliferation and chondrogenic differentiation of the given SMSCs and encouraged cartilage regeneration, thereby considerably enhancing the healing of cartilage defects.

Some other examples of chitosan-based scaffolds used for 3D bioprinting are summarized in Table 2. Overall, these studies suggest the potential of chitosan-based hydrogels in tissue engineering, drug delivery, and various other medical applications.

3.3 Biological characterization

3.3.1 Drug release. The porosity of the hydrogel makes a path for higher drug release. Depending on the application, a perfectly suitable drug has to be chosen.[166] writes that the drug should be selected in a way that improves stiffness, stability, and elastic properties. The selection of release media depends on the environment to be mimicked. Before release tests, the drug loading efficiency percentage must be calculated. The hydrogels were crushed by using a mortar and pestle.[21] The powdered hydrogel can be placed in the release media under continuous stirring until maximum release occurs. We can find the loading and encapsulation efficiency. Using the formulae below.

$$\text{Loading efficiency} = \frac{\text{Amount of drug in the hydrogel}}{\text{Weight of the hydrogel}} \times 100$$

$$\text{Encapsulation efficiency} = \frac{\%\text{Actual loading}}{\%\text{Theoritical loading}} \times 100$$

Once the loading and encapsulation efficiencies are determined, the *in vitro* drug release test can be performed using indirect or direct dissolution methods,[167] the loading method must be chosen carefully as it can directly influence the drug release. Direct immersion of hydrogel in buffer solutions[38] or Dialysis membrane bags can be used as an indirect method.[21] The beakers are placed in a dissolution shaker apparatus at a set rpm and temperature. This continuous agitation of beakers is to keep the sample from aggregating.[168] At specific time intervals, aliquots of the sample can be collected, and the same amount of fresh-release media amount of drug release can be calculated using a UV spectrophotometer. The corresponding cumulative drug release can be calculated using a calibration curve.

The porous structure of chitosan hydrogels allows for the sustained release of drugs from the framework. Guaresti *et al.* designed a pH-responsive Chloramphenicol-loaded chitosan hydrogel and measured the release of the drug.[169] They observed sustained drug release from the hydrogel, with the concentration increasing over time. The release process was slower than the swelling, and after 4 hours, the entire drug was released. The degree of cross-linking in the hydrogel and the hydrophilic/hydrophobic interactions will determine both the swelling capacity and the amount and rate of drug release.[170]

3.3.2 *In vivo* degradation. The presence of any foreign body for a while can cause harmful infections leading to cancer and sometimes death too. Hence it is vital to remove it in time as there cannot be a completely biocompatible material. The alternative for removal is *in vivo* degradation of the material/hydrogel with time.[74] has written that biodegradability can be tuned with different types of crosslinking agents, varying the concentration of crosslinkers. Biomaterials must be able to degrade to be safely, easily, and non-invasively removed from the body. With time the subsequent degradation of hydrogel provides space for new formation of tissues. To assess the hydrogel degradability *in vivo*, the self-healing injectable hydrogel solution was injected into the anesthetized animal body like mice, rats, guinea pigs, and rabbits. In general, ethyl ether[50,171–173] is used to anesthetize mice. The animal body can be exposed to UV to construct the gel *in situ*.[38] However, in ref. 172 the formation of transparent hydrogel under the skin was automatic without any additional trigger. In ref. 173, the hydrogel formation was confirmed by hand and observed using a digital camera.

The FDA has approved chitosan as a non-toxic, biocompatible, and biodegradable polymer for use in wound dressings.[174] Su F. *et al.* synthesized carboxymethyl chitosan-crafted polylactide (CMCS-PLA) and carboxymethyl chitosan (CMCS) hydrogel and assessed the biocompatibility and *in vivo* degradation of this prepared hydrogel.[175] The *in vitro* and *in vivo* studies demonstrated that the prepared chitosan hydrogels are biocompatible and degrade *in vivo* after 19 days, proving their promising future as a nanocarrier. A biodegradable carboxymethyl chitosan/oxidative hyaluronic acid hydrogel, synthesized by Xia L. *et al.*, demonstrated intriguing characteristics following intraperitoneal injection.[176] Analysis

of the biodegradation, distribution, and urine excretion of this synthesized hydrogel revealed rapid drug dispersion to the blood, kidneys, and liver, with minimal distribution to the spleen, lungs, and heart. The majority of the hydrogel was eliminated *via* urine within 84 hours post-injection, while the remaining portion was excreted as comparatively smaller molecular weight fragments within 36 hours post-injection. Following *in vivo* degradation, these hydrogels exhibit remarkable characteristics, posing no adverse effects on tissues.[177] Their biocompatibility makes them suitable for various biomedical applications. Moreover, studies indicate that chitosan-based hydrogels can potentially enhance cell proliferation and differentiation, thereby offering therapeutic benefits in tissue engineering and regenerative medicine.

3.3.3 Cell viability or cell proliferation. Cell-based assays are prominently used to screen collections of chemicals to observe the impact of test molecules on cell growth and cytotoxic effects that ultimately result in cell death. Regardless of the cell-based test employed, it is essential to understand how many viable cells are still present after the experiment. The number of cells that are still viable can be determined using a variety of assay techniques.[178] The resazurin reduction, protease activity, and tetrazolium reduction assay each measure a different aspect of general metabolism or an enzyme activity as a sign of live cells. To identify live cells, several tetrazolium compounds have been employed.[179] The most often used substances are XXT, MTS, MTT, and WST-1.[180] These cells along with cellular pH and osmotic pressure controllers, supplements like proteins, vitamins, carbohydrates, amino acids, growth factors, and antibiotics are[29] seeded according to cell count in a 96-well plate and allowed for cell culture for a certain period.[180] Serums constitute a conventional integration of amino acids, vitamins, carbon sources (*e.g.*, glucose), and inorganic salts. Then the self-healing hydrogel solution is added to the well plates and incubated again.[180] The total number of viable cells is calculated by multiplying the number of cells discovered in all four squares by 104 (to get the average cell number per mL), multiplied by 2 (to get the Trypan Blue dilution factor), and the original medium volume of the complete cell suspension. By dividing the total number of labeled cells by the total number of cells and multiplying the result by 100, the percentage of viable cells may be calculated. Cell viability of 80 to 95 percent indicates good cell culture. The other method consists of staining the sample with dyes like calcein-AM (green), Safranine followed by observation under an inverted fluorescence microscope. The cells can be numbered using the ImageJ software. The relative growth rate can be calculated by placing the 96-well plate in a microplate reader at a set absorbance value. The growth rate ratio is also known as the cell proliferation ratio.

In extension to cell culture, chitosan aids cell differentiation; cells acquire specialized structures and functions during development or in response to specific signals. Differentiation allows cells to transform from an undifferentiated state (stem or progenitor cells) into specific cell types with distinct characteristics and functions. Also, they change gene expression, morphology, and functionality to fulfill specific roles in the body. This process is tightly regulated and influenced by various factors, including growth factors, signaling molecules, and environmental cues. Cell differentiation is a fundamental process involving embryonic development, tissue regeneration, and the maintenance of tissue homeostasis.[181] used chitosan graphene oxide composite hydrogels for the differentiation of neuroblastoma cells.

Modi U. *et al.*, developed a 3D tumoroid model using chemically modified dextran-chitosan hydrogel. This 3D tumoroid model exhibits physiologically similar migration, proliferation, and invasive potential.[182] Another chitosan hydrogel synthesized by Kwon J. S. *et al.* provides a 3D substrate for the cell proliferation, attachment, and differentiation of rat muscle-derived stem cells (rMDSCs) in the presence of valproic acid (VA).[183] The literature has demonstrated that modified chitosan-based hydrogels promote cell proliferation and differentiation of Human retinal pigmented epithelial-1 (RPE-1) cells,[184] osteoblasts,[185] neural progenitor cells (NSCs),[186] rat muscle-derived stem cells (rMDSCs),[183] fibroblasts,[187] and human adipose-derived stem cells (hADSCs).[188]

3.3.4 Blood compatibility. Blood compatibility refers to a material's ability to interact with blood without causing adverse reactions. Assessing this is crucial for hydrogels, which are extensively used in biomedical applications. Methods for evaluating blood compatibility include hemolysis assays, platelet adhesion and activation tests, coagulation assays, complement activation assays, and *in vivo* studies.[189] These assessments measure the hydrogel's impact on red blood cells, platelets, blood clotting, immune response, and overall physiological compatibility, ensuring its safety for use within the circulatory system and other blood-contacting applications. As discussed, chitosan stands as an FDA-approved biocompatible polymer, with numerous studies consistently demonstrating its nontoxic and biocompatible nature upon interaction with blood components. Su F. *et al.*, assessed the biocompatibility of a hydrogel prepared from carboxymethyl chitosan polylactide (CMCS-PLA) and CMCS using the hemolysis test.[175] Samples of CMCS and CMCS-PLA did not induce hemolysis in the blood system. It is widely acknowledged that if the Hemolysis Rate (HR) value is less than 5%, biomaterials can be considered suitable for biological applications. The HR values for CMCS and CMCS-PLA were found to be 1.4% and 1.7%, respectively, indicating that both materials possess exceptional anti-

$$\text{Cell Viability} = \frac{\text{No. of live cells}}{\text{Total no. of cells}}$$

$$\text{Growth rate ratio} = \frac{(\text{Absorbance value at the given incubation time}) - (\text{Initial absorbance})}{(\text{Initial absorbance}) - (\text{Absorbance of the reagent at set wavelength})} \times 100$$

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Table 3 Different biological applications of chitosan hydrogels

Type of application	Type of chitosan – substrate used	Main findings	Ref.
Tissue regeneration	N-Carboxyethyl chitosan	Electroactivity and conductivity	200
	Carboxymethyl chitosan	(1) Cytocompatibility	34 and 201–203
		(2) Wet-tissue adhesion	
		Antimicrobial properties	
Wound dressing	Quaternized chitosan	(1) Can be applied on irregular and motion wounds	59 and 204
		(2) Tissue adhesion	
	Acetic acid modified chitosan	(1) Nontoxic	69, 134 and 205
		(2) Blood absorption & hemostatic capability	
	Adenine-modified chitosan (AC)	Hemostatic effect, reduced inflammation, regeneration of collagen	135
	Chitosan Hydrochloride	Anti-inflammation, skin repair	81
	PEG-Modified Chitosan	Hemostatic effect, Tissue adhesion	19
	Carboxymethyl chitosan	(1) Enhances angiogenesis, collagen secretion	206 and 207
		(2) Tissue adhesive	
Drug delivery	Orotic acid modified chitosan	Stimulus responsiveness, injectability, conductivity	126
Gastrointestinal	Carboxymethyl chitosan	Cell migration, wet tissue adhesive drug delivery	201
Cell carriers	N-Carboxyethyl chitosan	Cell delivery carrier for cell therapy for skeletal muscle repair	200 and 208
	Chitosan Hydrochloride	RPC-based transplantation therapy	209
		Proliferation & differentiation of RPC	
	PEG-modified chitosan	Cell therapy & cardiac tissue repair	210
	Carbamoylated chitosan	Improved cell viability, adhesion, growth and cell differentiation,	115 and 116
Cancer treatment	Acetic acid modified chitosan	Superior mechanical strength	134
	N,O carboxymethyl chitosan	Prolonged release of anticancer agents at the tumoral site	74

hemolysis qualities. Overall, literature suggests that chitosan-based hydrogels are biocompatible and suitable for biomedical applications.[190]

3.3.5 Antibacterial activity[191]. Antibacterial agents fight against infectious diseases, and hence to evaluate it, a small volume of Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) is added into the 24-well microplate consisting of the hydrogel solution. The suspension is evenly scattered on an agar plate and incubated at 37 °C for a day or so (18–24 h),[135] the number of colonies formed is counted, and the kill percentage can be calculated using the formula below.

$$\%\text{kill} = \frac{(\text{colony count of control}) - (\text{colony count of hydrogels})}{(\text{colony count of hydrogels})} \times 100$$

The other method is the zone of inhibition. The bacterial suspensions alone are spread on the Luria-Bertani agar plates and some amount of hydrogel samples were placed on the surfaces.[192] The same can be performed by making a hydrogel + bacterial suspension beforehand. After prolonged incubation,[193] a circular region will be created where the bacteria cannot grow around the samples; this was considered the inhibition zone. The zone of inhibition can be obtained using vernier calipers.[14] observed the morphology of bacteria in the hydrogel using a Scanning electron microscope after incubating for a period of 5 h. They found that the hydrogel with *S. aureus* cells revealed a wrinkled, ruptured, and damaged membrane structure and morphology, but the hydrogel with loaded *E. coli* did not significantly affect the cell morphology.

Chitosan-based injectable, self-repairing hydrogels exhibit remarkable antimicrobial efficacy, particularly against drug-resistant bacteria, owing to their multifaceted antimicrobial mechanisms.[194] These hydrogels possess inherent cationic properties, enabling electrostatic interactions with negatively charged bacterial cell membranes, thereby disrupting membrane integrity and inducing leakage of cellular contents.[195] Additionally, chitosan-based hydrogels can interfere with bacterial cell wall synthesis, inhibit microbial enzyme activity, and disrupt microbial biofilm formation, further augmenting their antimicrobial potency.[196] Moreover, the injectable and self-repairing nature of these hydrogels allows for targeted delivery and sustained release of antimicrobial agents, ensuring prolonged therapeutic efficacy against recalcitrant bacterial strains.[197] Pengpeng Deng *et al.*, developed injectable, self-healing chitosan hydrogels modified with adenine for use in wound healing applications. The hydrogels shown remarkable antibacterial properties against drug-resistant bacteria, fungus, and Gram-positive and Gram-negative bacteria. Furthermore, these designed hydrogels significantly improved wound healing and decrease the infiltration of inflammatory cells in skin defect model.[198] In another study, Farasati Far B. *et al.*, developed gelatin corss-linked chitosan-based hydrogels and antibacterial efficiency was examined against Gram-positive and Gram-negative bacteria.[199] These antibacterial studies demonstrated that prepared chitosan hydrogels significantly inhibit bacterial growth and reduce the MIC and MBC values against *E. coli* and *S. aureus*. The biocompatibility of the hydrogels was also confirmed by the MTT assay and demonstrated antibacterial effects of chitosan-based hydrogels.

Overall, chitosan-based hydrogels could be a promising avenue for use in antibacterial applications.

4. Biological applications of chitosan-based hydrogels

Chitosan-based hydrogels offer promising avenues in biological applications due to their biocompatibility and bioactivity. These hydrogels find utility in tissue engineering, wound healing, and drug delivery systems, providing a versatile platform for controlled release and cell interaction, thus advancing therapeutic interventions in regenerative medicine. Table 3 summarize the different biological applications of Chitosan hydrogels.

4.1 Tissue regeneration

Self-healing injectable hydrogels have emerged as an appealing technique for tissue regeneration in various medical applications. These hydrogels are composed of cross-linked polymers that can absorb huge quantities of water, giving them a gel-like consistency similar to extracellular matrix (ECM). They possess the unique ability to autonomously repair themselves upon damage, making them particularly advantageous for tissue engineering and regenerative medicine.

4.2 Cardiac tissue

Myocardial infarction is the world's largest reason for heart failure. Cardiac injuries are irreversible, and heart transplantation was the only solution till the invention of self-healing injectable hydrogels, but modified chitosan with self-healing injectable properties could reduce the infarct size, protect the transplanted cells and improve cardiac function[211] due to their ability to conjugate with various bioactive molecules.[212]

4.3 Cartilage tissue

Cartilage is a complex and vascular tissue with limited self-healing capacity. Injectable hydrogels can be loaded with chondrogenic cells, growth factors, and extracellular matrix components and then injected into the damaged cartilage site to provide a supportive environment for cell growth and tissue formation, while its self-healing properties ensure the integrity of the scaffold, promoting the regeneration of functional cartilage.[34]

4.4 Bone regeneration

chitosan-based self-healing injectable hydrogels can mimic the natural extracellular matrix, providing structural support and an ideal environment for bone regeneration in bone defect repair caused by trauma, disease, or surgical procedures.

4.5 Bone defect repair

Chitosan-based self-healing injectable hydrogels have been used to repair bone defects caused by trauma, disease, or surgical procedures. These hydrogels can be injected directly into the defect site, promoting cell proliferation, angiogenesis, and mineralization, leading to bone regeneration.[203] Chitosan-based injectable hydrogels can treat osteoarthritis, a degenerative joint disease[213] Periodontitis is an inflammatory disease caused by infection, destroying soft gum tissues and subsequent bone loss. Carboxymethyl chitosan (CMCS), which possesses properties of wet-tissue adhesion, excellent antibacterial capabilities, and cytocompatibility, can be utilized in this context. Additionally, CMCS holds the potential for drug delivery to wet tissues in oral diseases.[201] Other than the above applications, it can be used in nerve regeneration which is a gradual process involving the disconnection of nerve sheaths and underlying neurons. To expedite healing and regeneration, external assistance is required.[202] In this study, a conductive hydrogel was introduced into the cavity of a carboxymethyl chitosan conduit prepared via electrodeposition. The application of the hydrogel demonstrated the promotion of sciatic nerve regeneration in an in vivo setting. Skeletal muscle possesses the intrinsic regenerative capacity, enabling it to undergo regeneration in response to minor injuries, as well as more severe conditions such as myopathies, prolonged denervation, significant traumatic injury, ischemia, and aggressive tumor ablation.[200] These clinical conditions can result in substantial muscle loss.

4.6 Wound healing

The ability to be administered in a minimally invasive manner allows the hydrogel formulation prepared as a liquid can be easily injected into the wound site. Once it comes in contact with physiological conditions, the liquid transforms into a gel, forming a physical barrier that protects the wound from external contaminants and provides a moist environment for wound healing so that secondary infections do not occur. Chitosan has vast applications in wound dressing because of its hemostatic capability, antibacterial, antioxidant properties, and tissue adhesion. The uniform porous structure of chitosan-based hydrogels is beneficial in absorbing and storing excess exudate from the wound area and ensures oxygen permeability for wound healing. Its conductive nature can be exploited to monitor the real-time physical condition of the patient reducing the risk of delaying the disease.[18] The combined motion of polymer chains and the dynamic nature of Schiff bases within the hydrogels, particularly at the fracture surface, facilitate the re-crosslinking of free functional groups. This unique characteristic grants the hydrogels rapid self-healing capability. Consequently, the morphology and mechanical properties of the hydrogels can effectively recover following damage, showcasing their potential to withstand internal and external forces at the site of injury.

Chitosan has gained attention in wound healing due to its unique properties such as antimicrobial activity, haemostatic effect, and promotion of tissue regeneration.[214] When formulated into hydrogels, chitosan exhibits enhanced properties, making it an attractive material for various wound types.[215]

4.6.1 Diabetic wounds.
Diabetic wounds pose a significant challenge in wound management due to impaired healing processes.[216] Chitosan-based hydrogels offer a promising
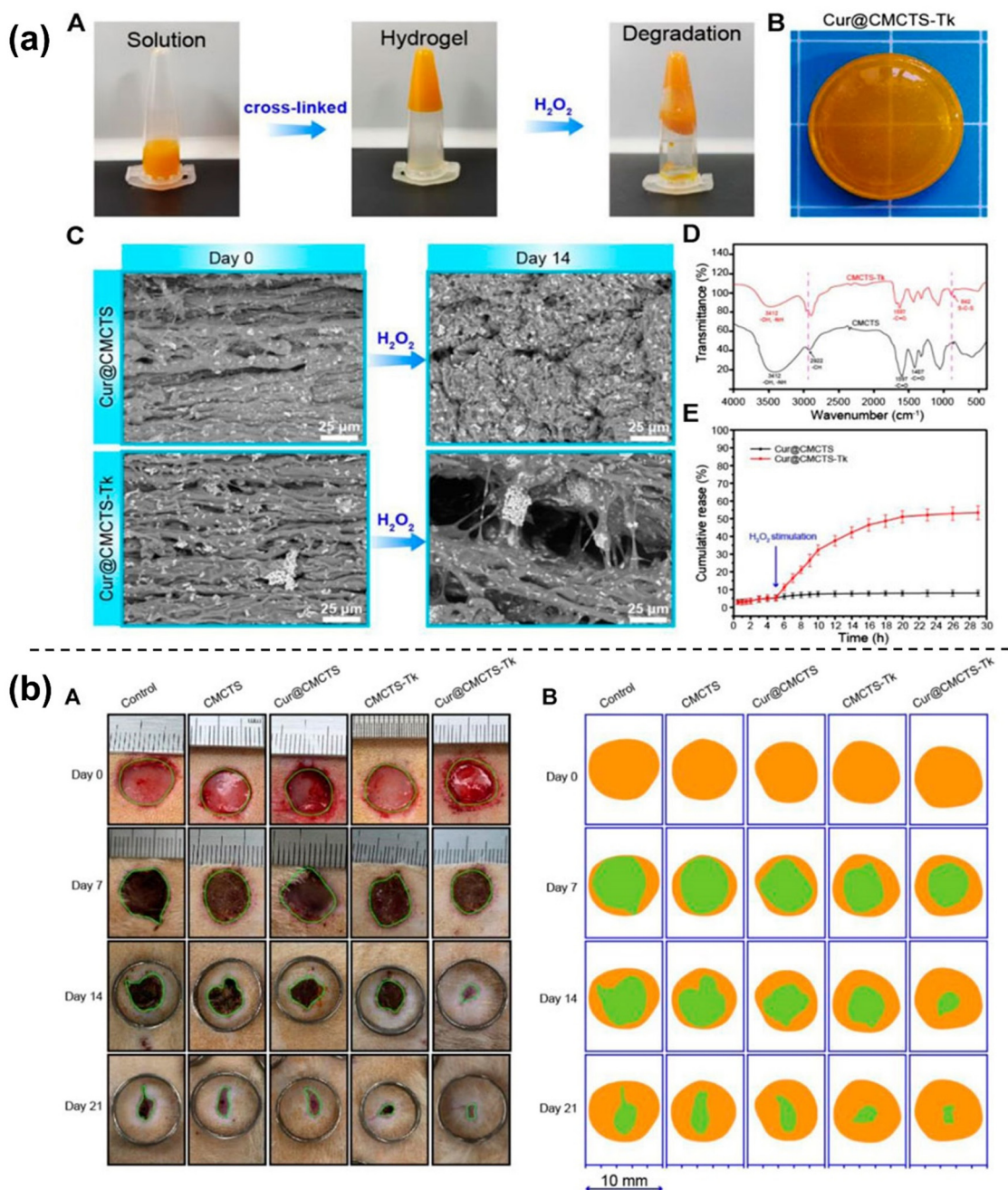
Fig. 9 Characterization including SEM analysis, FTIR and release study of prepared Cur@CMCTS-Tk hydrogel (a). Digital photographs of the wounds on the dorsum of rats treated with Cur@CMCTS-Tk hydrogel for up to 21 days (b). Figures reproduced from *Front. Pharmacol.* [ref. 18], Open access, Copyright © 2021, Frontiers.

solution by providing a scaffold for cell proliferation and angiogenesis. that chitosan hydrogel loaded with growth factors (such as EGF, Epidermal Growth Factor) accelerated healing in diabetic foot ulcers by promoting cell proliferation and collagen deposition, along with exhibiting antibacterial effects.[217]

4.6.2 Acute wounds. Acute wounds resulting from traumatic injuries require rapid and effective treatment to prevent infection and facilitate healing.[218] Chitosan-based hydrogels have been utilized as wound dressings due to their haemostatic properties and ability to adhere to the wound bed. Research by Zhang Y. *et al.*, and Guo S. *et al.*, demonstrated the efficacy of chitosan-based hydrogels in promoting haemostasis and accelerating wound closure in acute trauma cases, skin injury, rapid hemostasis and hemorrhage.[219,220]

4.6.3 Ulcerated wounds. Chronic ulcerated wounds, such as pressure ulcers and venous ulcers, present persistent challenges in clinical management.[221] Chitosan-based hydrogels offer a multifaceted approach to ulcer healing by reducing inflammation, promoting granulation tissue formation, and

preventing bacterial colonization.[222] An *in vivo* study conducted by J. H. *et al.*, on rabbits and pigs demonstrated significant improvements in gastrointestinal ulcer healing rates with the application of chitosan hydrogel containing EGF compared to control groups.[223] The EGF-chitosan hydrogels were applied in two models of rabbits and pigs: the acetic acid-induced gastric ulcer model (AAU) and the mucosal resection-induced gastric ulcer model (MRU). The ulcer size decreased up to 2.3 times within 3 days and 5.4 times within a single day in the AAU and MRU models, respectively, after ulceration implying the chitosan hydrogels as a promising candidate for the treatment of ulcer wounds.

4.6.4 Sports wounds. Athletes frequently encounter abrasions, lacerations, and other skin injuries during sports activities. Chitosan-based hydrogels provide an ideal wound dressing solution for sports-related wounds due to their flexibility, antimicrobial properties, and promotion of tissue regeneration.[224] Djekic, L. *et al.*, developed the chitosan-based hydrogels for the skin wounds leading to sustained release of ibuprofen.[225]

4.6.5 Burn wounds. Burn injuries often result in extensive tissue damage and impaired wound healing. Chitosan-based hydrogels offer several advantages in burn wound management, including cooling effects, moisture retention, pain reduction, infection prevention, and promotion of epithelialization.[226,227] Yang C. *et al.*, prepared carboxymethylated chitosan hydrogel cross-linked with thione groups and loaded with curcumin (Cur@CMCTS-Tk).[228] In the full-thickness skin burn defect rat model, this hydrogel loaded with curcumin effectively accelerated the wound healing process and demonstrated good regenerative properties, such as the formation of hair follicles, promotion of new blood vessel formation, and highly ordered collagen fiber arrangement (Fig. 9). Overall, because the produced hydrogel can scavenge excess ROS, it may be applied to wound healing and tissue regeneration.

4.7 Drug delivery

Hydrogels can be induced into the human body through different routes such as oral, transdermal, ocular,[64] rectal, vaginal,[229] and subcutaneous.[230] Oral delivery can treat the stomach, intestine, *i.e.*, Intraperitoneal, oral cavity, and colon.[231] Chitosan hydrogels can undergo significant swelling and degradation in aqueous environments. This can result in a loss of structural integrity over time, leading to decreased mechanical properties and potential release of encapsulated substances. The degree of swelling and degradation depends on factors such as chitosan concentration, degree of acetylation, pH of the surrounding environment, and the nature of encapsulated molecules.

pH sensitivity and thermal, photothermal responsive techniques are smart drug delivery applications.[126] Yongyan Yang *et al.*, 2023 synthesized a pH-responsive hydrogel with orotic acid-modified chitosan and 2,6 diaminopurine (OACS-DAP). When formed in an acidic solution, the hydrogel was in a solid state but at a pH above 4, led to a phase shift from solid to liquid or semi-liquid state. This physical change was reversible in nature depicting the self-healing ability of chitosan. Often pH-responsive drug release is induced in applications or gastrointestinal and Periodontal Diseases.[201] Additionally, self-healing hydrogels can be combined with other technologies, such as nanoparticles[232,233] or microneedles,[234,235] to further enhance drug delivery capabilities.[153] Marapureddy *et al.* 2022 induced nanosheets of graphene oxide to improve the mechanical strength and to obtain a pH-responsive sustained drug release. In addition to hydrogels[153] could fabricate chitosan films using the same. These hybrid systems can provide additional functionalities, such as improved drug loading capacity, enhanced targeting, or sustained release.

4.8 Cell carriers

Self-healing and injectable hydrogels offer distinct advantages compared to conventional injectable hydrogels for cell delivery: (1) mitigation of cell loss risks during injection: Self-healing and injectable hydrogels minimize the potential risks associated with cell loss during the injection process. They provide mechanical protection to the delivered cells, safeguarding them from shear damage during injection.[121] (2) Assurance of cell morphology and functionality: These hydrogels enable the confirmation of cell morphologies and functionalities within the three-dimensional (3D) microenvironment. This allows for quality control of loaded cells before transplantation, ensuring optimal cellular characteristics for successful outcomes.[236] (3) Facilitation of rapid mechanical recovery: Self-healing and injectable hydrogels can rapidly recover from mechanical damage. This property preserves the intrinsic functionalities of the hydrogel and extends the service life of implanted cell-loaded hydrogels. Maintaining the hydrogel's structural integrity ensures the sustained functionality and longevity of the encapsulated cells.[237]

5. Conclusions, drawbacks & prospectives

Self-healing injectable hydrogels exhibit the unique ability to withstand and reverse the damage that accumulates over time and external factors. They can be intentionally designed to mimic physiological conditions, but the absence of reversible bonds refrains them from recoverability. In a biopolymer like chitosan, introducing Schiff base linkages create reversible imine bonds, which can revert back the damage both macroscopically and microscopically. The insoluble nature of chitosan was addressed using different modifications, be it with periodate oxidation or poly-ethyl-glycol, a non-toxic, water-soluble polymer, or the usage of any chitosan derivatives. To determine the properties various instruments and experiments equipped in all the tests, chitosan could reveal its recoverability, printability, biocompatibility, and mechanical strength. As an application to these properties, chitosan was used in tissue-engineered scaffolds, wound dressings, drug delivery, and cancer treatment.

Chitosan can be used as a carrier for drugs, proteins, antigens, and genes, but the complication in insolubility requires harsh and long synthesis procedures to be followed. Modification of chitosan enhances the solubility and mechanical performance in neutral and alkaline solutions but might lower its inherent properties like biocompatibility and degradation. In fact, it is better to combine biodegradable substances as the slightest non-biodegradability *in vivo* has a sure chance of infection, cancer, and sometimes death too. They often need to be combined with strength-inducing substances because of their low strength. This limits its combination with various components, although it's compatible. Traditional hypodermic injections can cause pain and discomfort. The inherent property of the above-discussed hydrogels is injectability, but using hydrogel-forming microneedles (HFMs) provides a minimally invasive approach through transdermal layers eliminating the need for injection equipment.

It's worth noting that the specific drawbacks of chitosan-based hydrogels can be mitigated or overcome to some extent through modifications, such as blending with other polymers, incorporation of cross-linking agents, reinforcement with fibers,[36,238] or optimization of processing conditions. These modifications can improve mechanical properties, control gelation behavior, and enhance biocompatibility.

The Prospects of self-healing injectable hydrogels are promising and hold significant potential for various applications in the medicinal field. The tunable nature of self-healing injectable hydrogels allows for customization based on patient-specific needs. By tailoring the properties of these hydrogels, such as mechanical strength, degradation rate, and bioactive molecule release, they can be optimized for individual patients or specific medical conditions, supporting the concept of personalized medicine by encapsulating different drugs and therapeutics. Their potential to revolutionize minimally invasive procedures makes them both patient and doctor friendly. (1) Inculcating the conductive nature of chitosan-based self-healing injectable hydrogels can aid in biosensing applications. (2) Recently, chitosan-based injectable microneedles were invented whose application was safe and easy. It requires a gentle pressure or patching onto the skin without any specialized equipment or medical professionals. In the same route, other polysaccharides can be designed for various and better applications.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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