Milk protein-based hydrogels: Development and biomedical applications

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ABSTRACT

Hydrogels are an advanced class of biomaterials with similar properties to living tissues. Several polymers have been investigated for the preparation of hydrogels that closely mimic the structural and functional properties of the extracellular matrix. Proteins with easily modifiable functional groups, specific biochemical effects, and sensitivity to external stimuli are promising candidates for the preparation of hydrogels for biomedical applications. Among them, natural milk proteins, due to their high yield, high-quality control, low cost, and certain biological properties, have become a major focus of research. However, there is a lack of comprehensive reviews focusing specifically on milk protein-based hydrogels. Here, we synthesise the developments in milk protein-based hydrogels, focusing primarily on hydrogels derived from milk proteins. We described the methods used to construct milk protein-based hydrogels and summarised advances in representative applications of milk protein-based hydrogels, such as controlled delivery and regenerative medicine, as well as related preclinical animal experiments and an exploratory clinical pilot study. Finally, we discuss the prospects of milk protein-based hydrogels in biomedical applications. We anticipate that this review will serve as a theoretical basis for the biomedical use of milk proteins and provide a reference for their continued development.

Keywords:
Biomedical application; Construction method; Hydrogel; Milk protein

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1. Introduction

Biomaterials play an important role in the biomedical field as drug-releasing stents, wound dressing materials, sutures, vascular grafts, hip joints, implants, contact lenses, and several other biomedical devices.[1] Recent developments have provided the ability to control material properties with great success in the fields of therapeutic medicine and tissue engineering.[2,3] Currently, hydrogels have received extensive attention as an important class of materials for biomedical applications due to their excellent biocompatibility, high water content, multifunctionality, and diverse forms of application.[4-8] Hydrogels are elastic three-dimensional (3D) polymer networks that are held together by hydrogen, ionic, or covalent crosslinking bonds and do not dissolve in liquids.[9] Since the introduction of hydrogels as biomaterials in the 1960s,[10] they have found numerous applications in controlled delivery, regenerative medicine scaffolds, bio-inks.[11-14] Notably, the physical and mechanical properties of hydrogels are closely resemble those of biological tissues.

Hydrogels can be categorised into synthetic hydrogels and natural hydrogels according to their sources. Synthetic hydrogels have good mechanical strength and excellent water-absorbing capacity, but their use is limited by certain unpredictable aspects of their toxicity, and their shortcomings in terms of poor biodegradability and biocompatibility that may also pose environmental problems.[15] In contrast to synthetic hydrogels, natural hydrogels, which are composed of natural substances such as proteins and polysaccharides, have received much attention because they can produce functional colloids rapidly and are safe for humans and environmentally friendly.[16] Among

them, proteins have an advantage over polysaccharides in making hydrogels because they possess more functional groups available for modification (amino, thiol, hydroxyl, and carboxyl groups), which are more sensitive to external stimuli, have the unique ability to recognize certain peptides, and can self-assemble.[17,18] In addition, as the fundamentally important macromolecules in living systems, proteins have evolved to perform very specific biochemical roles.[19,20] The biomaterials prepared from them can, therefore, be expected to retain these functions.[21-23] Several proteins have been evaluated for their performance as biomaterials (**Table 1**).[8,24-42]

Among various proteins, milk proteins occupy a special position based on their functional properties. Milk proteins make up about 3.5% of the total milk composition and include different casein and whey proteins (WP). Casein constitutes 80% of milk proteins and consists of $\alpha_{S1\text{-series}}$-, $\alpha_{S2\text{-series}}$-, β- and κ-casein. They are bound together by protein-protein interactions (hydrophobic, hydrogen, and electrostatic binding) and by the presence of calcium and phosphate (6-8% by weight).[43] WP is extracted from the liquid portion of milk remaining after the milk has coagulated and the casein has been removed during cheese production.[44] WP makes up 20% of milk proteins and includes alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG), bovine serum albumin (BSA), lactoferrin (LF), immunoglobulins and lysozyme. It has been shown that WP is richer than casein in essential amino acids, including sulphur-containing amino acids and branched-chain amino acids.[45] Milk proteins have unique advantages over other natural protein polymers such as high yield, high-quality control, and low price. Moreover, milk proteins have also been found to have specific physiological functions, such as antioxidant,[46] antimicrobial,[47] antiviral,[48] immunostimulant,[48] and anticancer[49] potentials. Based on these excellent properties, researchers are interested in the application of milk proteins. Several groups are exploring milk protein-based materials including carbon quantum dots,[50] microspheres,[51] nanoparticles,[52] fibres,[53] and hydrogels[54] for different biomedical applications. However, hydrogels constructed from milk proteins combine the advantages of milk proteins and hydrogels and show promising applications in controlled delivery[55] and regenerative medicine.[39]

This review describes the potential of milk protein-based hydrogels. Milk proteins have been widely recognised as an interesting biomaterial. Although there is a good amount of literature available on general properties and applications of milk protein-based biomaterials, there is a lack of extensive review articles that specifically focus on milk protein-based hydrogels. Milk protein-based hydrogels have a strong potential to be utilised in biomedical applications. Our work is an effort to highlight the research that has been done in the area of milk protein-based hydrogels. It describes several methods that have been used to construct milk protein-based hydrogels, as well as the biomedical applications of these hydrogels (**Figure 1**). It aims to provide an overview of the advances that have been made and the future courses available. It will provide an overview of the milk protein-based hydrogels as well as direct the readers to the specific areas of application.

2. Search strategy

For this review, electronic searches were conducted in the Web of Science and PubMed databases to identify literature on the construction methods of milk protein-based hydrogels and their biomedical applications, published before November 2024. The search terms used were: (milk protein [Topic]) AND (hydrogel [Topic]). The results were further screened by title and abstract to exclude irrelevant studies and those in which milk proteins were not the primary material for hydrogel construction. Ultimately, 122 articles were selected for inclusion in this review.

3. The structures of milk proteins

Understanding the structure of milk proteins is essential for the preparation of milk protein-based hydrogels and their related applications. In addition to the differences caused by batch variations, milk proteins from different species may exhibit variations in their structure and properties.[56] For example, as described by Park *et al.*,[57] micelle structures in goat and sheep milk differ in average diameter, hydration, and mineralisation from those of cow milk. Caprine casein micelles contain more calcium and inorganic phosphorus, are less solvated, less heat stable, and lose β-casein more readily than bovine casein micelles. In this review, milk proteins from cows were used as the subject of study unless otherwise stated. **Figure 2** presents the structures and basic properties of milk proteins.[58] Casein is a non-globular protein mixture in which the molar ratios of $\alpha_{S1\text{-series}}$-, $\alpha_{S2\text{-series}}$-, β- and κ-casein are approximately 4:1:4:1. These four protein fractions are amphiphilic, with molecular weights ranging from 19 to 25 kDa and pI ranging from 4.1 to 5.3.[59] Tyrosine and lysine are amino acids that are abundant in casein. The casein molecule lacks a tertiary structure and is present in milk in the large structure of casein micelles. Each micelle consists of approximately 20,000 casein molecules, forming a porous network of non-spherical particles.[60] The interior of these micelles contains a large number of water molecules, between 3 and 4 g of water bound per gram of protein, which is an important feature for maintaining the internal structure of casein micelles. Meanwhile, inorganic mineral factors,

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Table 1. Several proteins that have been used to prepare hydrogels

Protein	Functions in nature	Advantage	Disadvantage	Application	Reference
Collagen	Principal protein of the extracellular matrix that offers structural support	Good biocompatibility, promotes cell adhesion	Low mechanical strength, rapid degradation, high cost	Delivery of growth factors, tissue engineering, corneal replacement, bone regeneration, cartilage repair, angiogenesis, cardiac therapy, gene therapy	8, 24-26
Gelatine	Collagen in its degraded form	Good biocompatibility, promotes cell adhesion, lower immunogenicity as compared to collagen, forms gels by temperature change	Low mechanical strength, rapid degradation, heat sensitivity	Delivery of growth factors, tissue engineering, differentiation of pluripotent cells, bone regeneration, cardiac therapy, wound repair	27-30
Silk fibroin	Basic ingredient for silkworm traps	Good biocompatibility, high mechanical strength	Lack of cell adhesion site, limited solubility	Delivery of therapeutic molecules, tissue engineering, bone regeneration, cartilage repair, cardiac therapy	31-34
Fibrin/ fibronectin	Component of the extracellular matrix	Good biocompatibility, promotes cell adhesion, very closely mimics the properties of the soft tissues	Limited mechanical strength, high cost	Tissue engineering, angiogenesis, cardiac therapy, wound repair	35-38
Milk protein	Composed of casein and whey proteins	Good biocompatibility, low cost, high productivity, suitable for probiotic encapsulation	Low mechanical strength, lack of cell adhesion site	Delivery of therapeutic molecules, tissue engineering, bone regeneration, cardiac therapy, wound repair	39-42

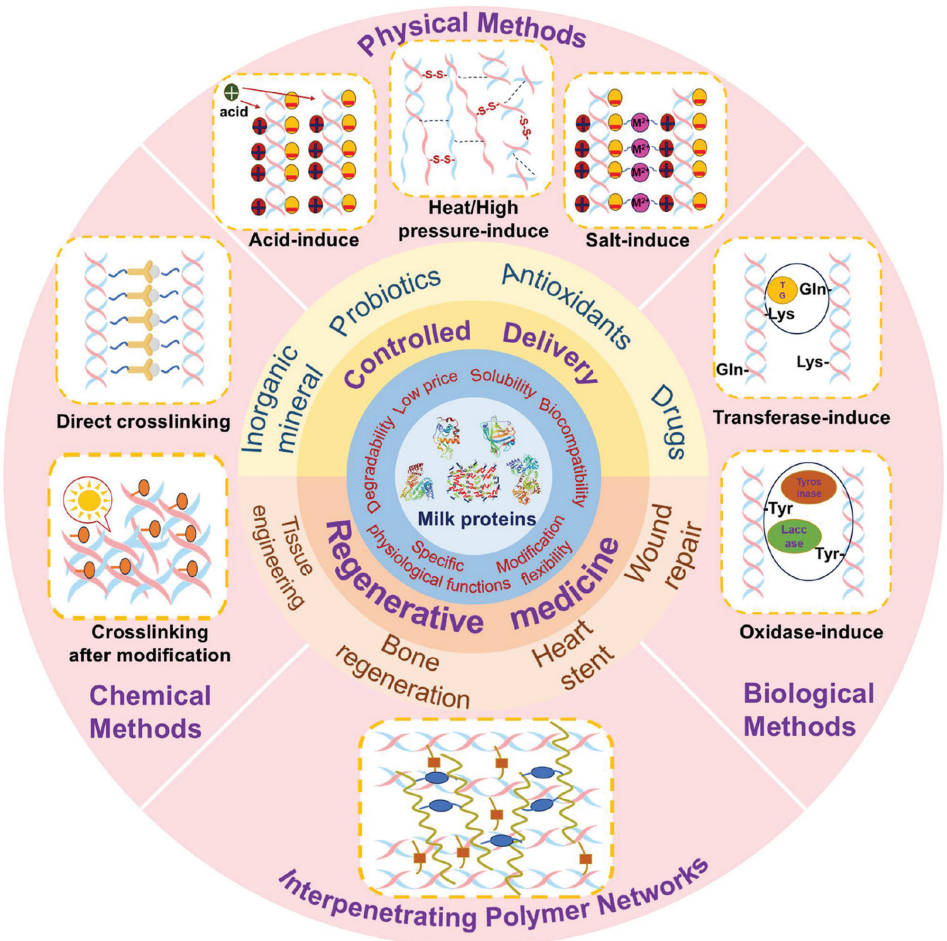

Figure 1. Scheme of the construction methods, properties, and biomedical applications of milk protein-based hydrogels. Created with Procreate 5.3.13 and PowerPoint 2021. Abbreviations: Gln: Glutamine; Lys: Lysine; M: Inorganic mineral; TG: Transglutaminase; Try: Tryptophan.

αs-casein and β-casein together constitute the internal structure of casein micelles, while κ-casein covers the surface layer of casein micelles, which is one of the important factors in maintaining the stability of casein micelles.[61]

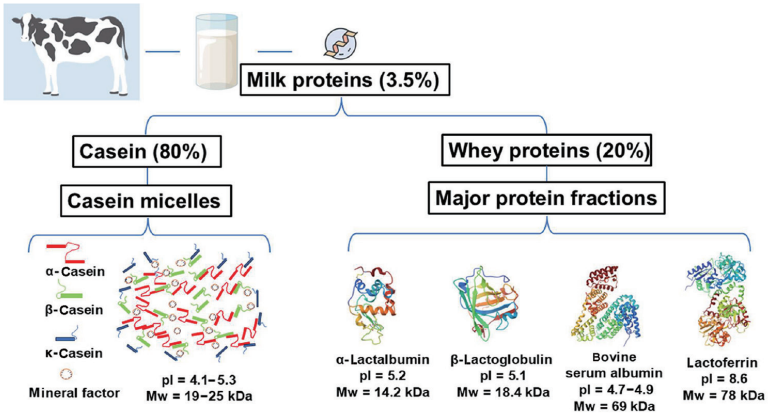

Figure 2. Schematic diagram of the structures and properties of milk proteins. Reprinted with Abaee *et al.*[58] Copyright 2017, Elsevier Ltd.

WP has a globular structure in which nonpolar, polar, and charged groups are evenly distributed.[58] β-LG is the major protein in WP, accounting for about 50% of the total WP. It consists of 162 amino acid residues, with a molecular weight of about 18.4 kDa and a pI value of 5.1.[62] Leucine, alanine, glutamic acid, and lysine are the amino acids present in high content in the β-LG molecule. The molecular structure of β-LG consists of two complementary regions: the α-region and the β-region, which interact with each other to form the aggregation state. In addition, β-LG contains five cysteines that form two disulfide bonds. The structure of β-LG is influenced by the folding and aggregation effects of the two disulfide bonds. α-LA is the second largest protein in WP, accounting for about 20% of the total WP.[63] It is a calmodulin composed of 123 amino acids and is a strong calcium ion-binding protein. Its molecular weight is about 14.2 kDa with a pI value of 4.2. Lysine, leucine, threonine, and tryptophan are the amino acids present in high content in the α-LA molecule.[64] BSA accounts for about 6% of the total WP, and consists of 582 amino acid residues, with a molecular weight of 69 kDa and a pI value of about 4.7–4.9.[65] BSA consists of a globular protein formed by the folding of a polypeptide chain. The surface of BSA contains a large amount of glutamic acid, aspartic acid, lysine, and arginine. In addition, BSA contains 35 cysteines that form 17 pairs of disulfide bonds.[66] LF is one of the minor proteins in WP. It is a single-chain polypeptide characterised as an iron-binding glycoprotein.[67] LF has a molecular weight of approximately 78 kDa and consists of 691 amino acid residues, with a pI value of 8.6. LF contains 17 disulfide bonds and several amino acid residues, including cysteine, tyrosine, and methionine, among others.[68] Others, like immunoglobulins and lysozyme, are also minor proteins in WP. Immunoglobulin consists of two heavy chains (50–70 kDa) and two light chains (25 kDa) bound together by disulfide bonds.[69] Lysozyme is a single peptide chain consisting of 129 amino acid residues, three of which are cysteines and eight of which are tyrosines.[70] Its molecular weight is about 15 kDa and it is a heat-insensitive alkaline protein. The structures of milk proteins impart good biocompatibility, biodegradability and solubility, in addition to their excellent nutritional value.[71] Future research on the structure and features of milk proteins will be further explored by researchers through evolving technologies, this will allow for a deeper understanding of the complex interactions within milk proteins and their potential applications in the biomedical field.

4. The construction methods of milk protein-based hydrogels

The construction methods of milk protein-based hydrogels can significantly impact the biological and mechanical properties. The primary reason is the difference in the type and amount of interaction forces between chains in hydrogels synthesised by different construction methods.[72] Milk proteins contain a large number of hydrophilic functional groups such as carboxyl (-COOH), amino (-NH_2), hydroxyl (-OH), and peptide bonds (-CO-NH-). Therefore, there are numerous methods for preparing milk protein-based hydrogels. Most of these construction methods are realised by directing the reactions of denaturation and partial unfolding of milk proteins through covalent bonding (inter- and intramolecular disulfide bonding) and a wide range of non-covalent interactions (e.g., hydrogen bonding, ionic and hydrophobic interactions). Based on the treatment, we can broadly categorise these construction methods into physical methods, chemical methods, and biological methods. **Table 2** summarises the various construction methods used to prepare milk protein-based hydrogels.[39,41,73-101]

4.1. Physical methods

Physical methods are widely used to construct natural hydrogels. In this part, physical methods refer to physical procedures without adding additional chemical or biological components. These processes induce specific groups on the side chains of protein molecules to link together to form larger molecules, leading to the crosslinking of milk proteins. The crosslinking is not only caused by the formation of non-covalent bonds, but also by the formation of covalent bonds. Among them, covalent bonds are mainly disulfide bonds, while non-covalent bonding forces include electrostatic interactions, hydrogen bonding, and molecular chain entanglement. Physical construction methods include heat-induced aggregation, high-pressure induced crosslinking, acid-induced crosslinking, and salt-induced crosslinking (**Figure 3A**).

Table 2. Summary of each construction method for the preparation of milk protein-based hydrogels

Construction method		Principle	Advantage	Disadvantage	Optimal application	Reference
Physical method	Heat-induced crosslinking	Thermal aggregation process promotes the formation of covalent (disulfide) and non-covalent bonds (hydrophobic and electrostatic interactions)	High mechanical strength, safe and stable, low cost	Prone to excessive aggregation, not suitable for direct encapsulation temperature sensitive substances	Bone regeneration	73-77
	High-pressure-induced crosslinking	High-pressure promotes the formation of covalent (disulfide) and non-covalent bonds (hydrophobic and electrostatic interactions)	Softer and smoother texture, hindering the browning reaction, compared to heat-induced crosslinking	Low mechanical properties, requires special equipment, not suitable for direct encapsulation of pressure sensitive substances	Controlled delivery	78-80
	Acid-induced crosslinking	Reduced electrostatic repulsion between proteins and subsequent conversion of protein surface sulfhydryl groups to disulfide bonds	Adjustable mechanical properties, safe and stable, low cost	Not suitable for direct encapsulation of pH sensitive substances	Controlled delivery	81-84
	Salt-induced crosslinking	Salt ions shield electrons between proteins by forming salt bridges	As a carrier for inorganic minerals, safe and stable, low cost	Low mechanical properties, uneven structure	Controlled delivery	85, 86
Chemical method	Direct crosslinking	Chemical coupling reactions, formation of Schiff bases, amide bonds, etc.	High mechanical strength, fast reaction	High specificity, prone to toxicity	Controlled delivery	87-89
	Crosslinking after modification	Covalent bond formation by photoinitiation-generated radical polymerisation after milk protein modification	*In situ* formation, adjustable mechanical properties	Cumbersome modification process, susceptible to inflammation and toxicity	Wound repair	39,41,90,91
Biological method	Transferase-induced crosslinking	Transglutaminase catalyses the formation of ε-(γ-glutamyl)-lysine peptide bonds between glutamine and lysine residues of milk proteins	Safe, low cost	Low mechanical properties, poor stability	Controlled delivery	92-94
	Oxidase-induced crosslinking	Laccase catalyses the formation of free radicals from milk protein phenols which interact with each other to form disulfide and di-tyrosine bonds	High mechanical properties and water retention capacity, safe	High cost, requires small molecule phenolics, poor stability	Controlled delivery	95-97
		Tyrosinase oxidises tyrosine residues to form quinone intermediates, which further form covalent bonds with other residues such as C-N, C-C, and C-S	Adjustable mechanical properties, no need for small molecule phenolic assistance, safe	High cost, substrate limitations, poor stability	Controlled delivery	98,99
Interpenetrating polymer network		Multiple forces of physical and chemical interactions	Dual network structure, high mechanical properties	With some toxicity	Tissue adhesives	100,101

4.1.1. Heat-induced crosslinking

Heat-induced hydrogels are generated by the unfolding of polypeptide chains exposing initially buried hydrophobic amino acid residues, followed by self-polymerisation of protein molecules into a 3D network that traps water by capillary forces.[102] As shown in **Figure 3A1**, heat-induced crosslinking involves thermal denaturation and thermal aggregation processes.[103] The thermal denaturation process involves the unfolding of the molecules and the loss of helical structure.[104] When heating is continued, the thermal aggregation process begins, which involves the formation of disulfide bonds (covalent bonding) and other intermolecular interactions (non-covalent bonding) that bring the molecules into aggregation. If the protein concentration is equal to or higher than the critical minimum concentration required to form a gel network, the aggregates can form gels with a 3D network structure.[105]

During the formation of heat-induced milk protein-based hydrogels, casein tends to require higher temperatures to reach the thermal aggregation temperature due to its stabilised micelle structure. Beyer *et al.*[106] found that when the temperature exceeded 100°C, casein micelles entered the thermal aggregation process to form casein hydrogels. Heat-induced crosslinking of

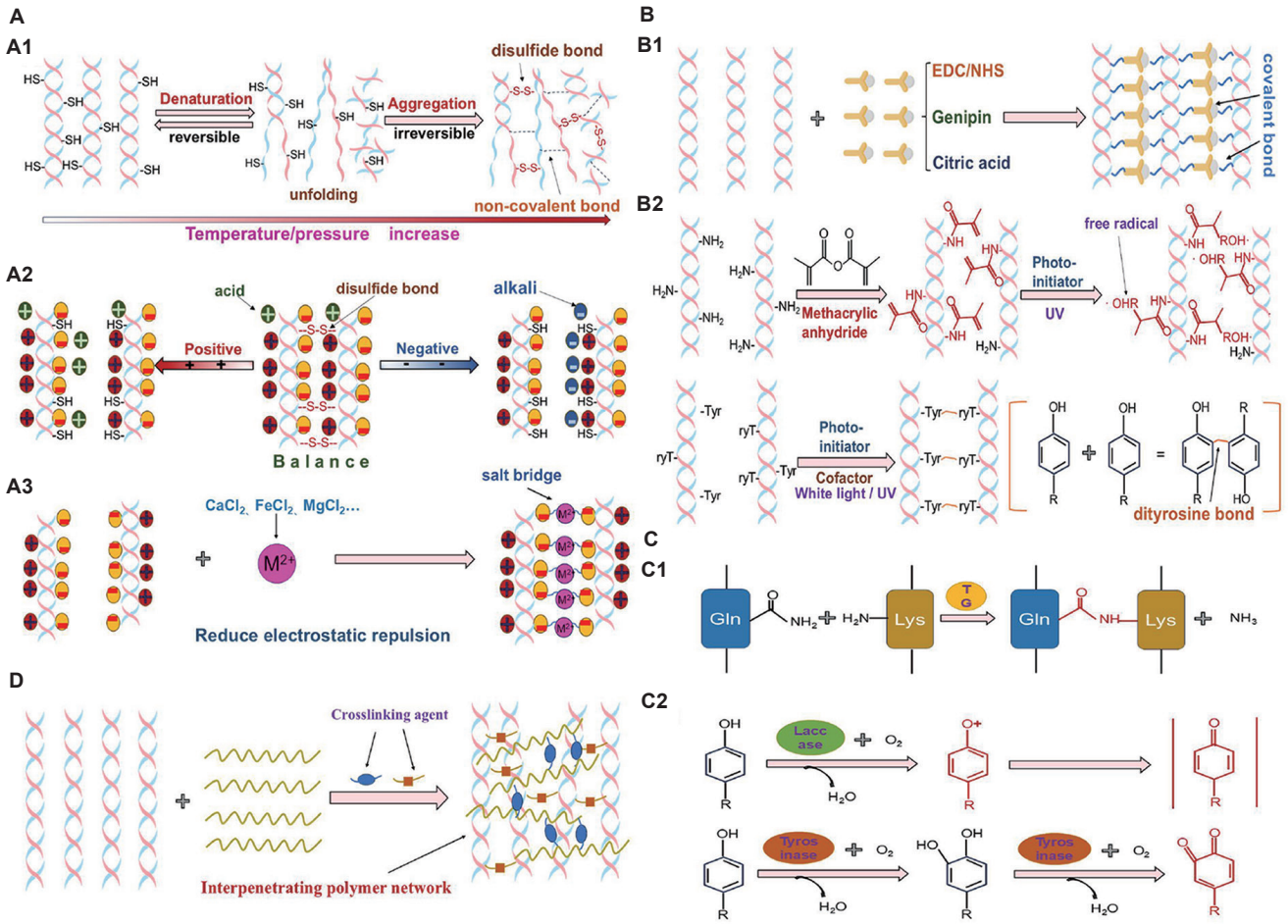

Figure 3. Schematic diagram of the construction methods of milk protein-based hydrogels. (A) The mechanisms of physical methods. (A1) Heat and high-pressure induced crosslinking. (A2) Acid-induced crosslinking. (A3) Salt-induced crosslinking. (B) The mechanisms of chemical methods. (B1) Direct crosslinking. (B2) Crosslinking after modification. (C) The mechanisms of biological methods. (C1) Transferase-induced crosslinking. (C2) Oxidase-induced crosslinking. (D) The synthesis route of interpenetrating polymer networks. Created with Procreate 5.3.13 and PowerPoint 2021. Abbreviations: EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; Gln: Glutamine; Lys: Lysine; M: Inorganic mineral; NHS: N-hydroxy succinimide; SH: Sulphydryl; TG: Transglutaminase; Try: Tryptophan; UV: Ultraviolet.

WP is usually thought to be driven by β-LG.[73] Mulvihill *et al.*[74] first described the mechanism of thermal aggregation of β-LG and proposed that β-LG undergoes an irreversible aggregation reaction at > 70°C. However, α-LA, which lacks the sulfhydryl group, is highly thermally stable, and thermal induction does not cause it to aggregate sufficiently to form gels.[75] This is the same conclusion as Gezimati *et al.*[76] For BSA, on the other hand, the thermal aggregation temperature is > 62°C, as the secondary structure of BSA unfolds easily during heating. At 80°C, the aggregation rate of BSA is 50- to 100-fold faster than that of β-LG.[77]

However, it is important to remember that since this type of hydrogel requires heat treatment, the application of heat-induced gels is limited to formulations that do not contain heat-sensitive bioactive ingredients. Moreover, it has been noted that the temperature of heat-induced aggregation should not be too high. High-temperature treatment leads to an increase in disulfide bonds and a decrease in non-covalent bonds, which promotes excessive aggregation of proteins and the formation of larger aggregates.[107] Such larger aggregates reduce the continuity of the gel network and decrease the number of protein molecules connected to its surface, which leads to a decrease in the thermal stability and textural properties of the hydrogel. Therefore, controlling the temperature is beneficial for heat-induced hydrogels to produce aggregates with appropriate size and homogeneity, thus improving the thermal stability and gel properties.

4.1.2. High-pressure-induced crosslinking

Both heat and pressure are known to denature proteins, leading to aggregation and gelation.[108] Unlike thermal denaturation (which primarily disrupts hydrogen bonds), pressure denaturation of proteins involves the breakdown of hydrophobic and electrostatic interactions, due to a reduction in the volume of the protein solution.[109,110]

Casein and WP respond differently to high-pressure processing due to differences in amino acid sequence and conformation as well as their ability to establish intermolecular covalent and non-covalent bonds. In this section, WP hydrogels are mainly involved in the formation of disulfide bonds. Under pressure, globular WP denatures and unfolds, presenting reactive sulfhydryl groups, and aggregation is enhanced by disulfide

bonds, resulting in gelling.[111] In contrast, casein hydrogels are primarily non-covalent bond forming. Casein micelles break down into sub-micelles, and casein monomers during the pressure build-up phase. During the subsequent pressure release phase, micelle fragments, micelles and monomers aggregate and form new hyper-structures, culminating in gelling.[112] Fertsch *et al.*[78] compared the changes in the hardness of high-pressure (600 MPa, 30°C) induced hydrogels of casein and WP as a function of pressure retention time and pressure release rate. The authors found that the hardness of pressure-induced WP was mainly influenced by the degree of denaturation of WP and determined by the number of disulfide bonds, which occurred mainly during the holding time (approximately 70% at 30 minutes of holding compared to 63.4% at 15 minutes). In contrast, the hardness of pressure-induced casein hydrogels was primarily influenced by the rate of pressure release. In addition, the hardness of pressure-induced WP hydrogels was greater than that of pressure-induced casein hydrogels since WP hydrogels are formed by covalent bonding.

However, although the principle of high-pressure-induced crosslinking is similar to that of heat-induced crosslinking (both involving physical denaturation and aggregation), there are differences in the hardness of the two hydrogels. Van Camp and Huyghebaert79 compared the rheological properties of high-pressure induced (400 MPa, 30 minutes) and heat-induced (80°C, 30 minutes) WP concentrate hydrogels and showed that high-pressure hydrogels produced larger pore sizes and smaller G' and G" values because of the less intermolecular crosslinking. High-pressure treatments result in more soluble aggregates due to the breakage of covalent bonds, producing more and smaller peptides. As a result, the high pressure-induced hydrogels were more sensitive to protease activity than the heat-induced hydrogels and exhibited higher digestibility in the gastrointestinal tract.[80]

4.1.3. Acid-induced crosslinking

Under acid treatment, the electrostatic charge carried by the protein molecules gradually decreases as the pH approaches the isoelectric point of the milk proteins. This brings the protein molecules closer to each other, allowing the aggregates to form the initial protein network structure through physical forces.[113] Afterwards, sulfhydryl groups exposed on the protein surface form disulfide bonds between protein molecules through sulfhydryl-disulfide bond conversion and self-oxidation, which formed stable milk-protein-based hydrogels114 (**Figure 3A2**).

Both acid-induced casein and WP hydrogels are obtained by lowering the pH of the solution system. Nevertheless, during the acid-induced crosslinking process, the thermal denaturation pretreatment of globular WP is necessary to expose some hydrophobic groups or regions within the molecule to the surface. After the solution is cooled, a WP hydrogel is formed by lowering the pH.[115] Whereas this pretreatment step is not required for casein that presents a non-spherical micellar structure.[116] Tan and Joyner81 prepared acid-induced casein hydrogels at different pH values (2.3, 3.6, and 4.8) and found that the viscoelasticity of hydrogels decreased with the decrease in pH value. As the pH moved away from the casein isoelectric point (4.6), the repulsive forces between aggregates increased, resulting in fewer disulfide bonds and larger pores in the casein network, which led to looser structures and weaker gels. Ju and Kilara82 arrived at the same point by studying the pH changes and mechanical properties of WP hydrogels. However, it has been noted that at pH ~ pI, the solution produces larger aggregates (ranging from a few hundred nm to > 1000 nm), which promotes the formation of opaque, low water-holding capacity particulate gel networks.[117] In contrast, under the opposite conditions, pI < pH < pI, hydrogels that are transparent, have high water-holding capacity, and exhibit orderly arranged chain gels.[118,119] The orderly arranged chain gels were formed by the polymerisation of denatured proteins into curved, flexible chain-like structures (pH > pI) or the formation of rigid starch fibrillar structures (pH < pI), which correlated with the percentage of α-helices at different pH conditions.[83,84,120]

4.1.4. Salt-induced crosslinking

Salt-induced crosslinking is also one of the more common physical construction methods.[121] As shown in **Figure 3A3**, the addition of salt ions can usually lead to the aggregation of protein aggregates and eventually to gel formation by shielding electrostatic charges or hydrophobic interactions of specific ions. Since casein already contains calcium phosphate in its structure, the addition of salt ions does not seem to enable the crosslinking of casein molecules. During salt-induced WP hydrogel formation, proteins are thermally denatured to expose the hydrophobic groups in their molecules, and the protein condensate formed relies on the electrostatic repulsive forces acting between them to form a stable dispersion system.[122] $CaCl_2$[123] and NaCl[124] are the most common salts used in the salt-induced crosslinking of WP hydrogels, in addition to $FeCl_2$,[125] $MgCl_2$,[126] $MnCl_2$,[127] $ZnCl_2$[127] and others.

However, it has been noted that there is a significant difference in the ability of different salts to induce milk protein crosslinking. Veerman *et al.*[85] specified the differences in the mechanism of β-LG crosslinking induced by monovalent and divalent salts. The authors found that divalent salt ions, in addition to the above effects, could form salt bridges between proteins (e.g., protofibrils-—Ca^{2+}—protofibrils-) interacting with negative ionic groups in the protein aggregates, which is more conducive to the formation of hydrogels. Similar conclusions were shared by Marangoni *et al.*[86] Marangoni *et al.* further observed the aggregate structure and found that NaCl-induced WP hydrogels, both small and large aggregates formed slowly. In contrast, CaCl2 induced WP hydrogels, the formation of smaller aggregates was slower, while the formation of larger aggregates was relatively faster, which could promote the formation of gels. Significant differences in the mechanism and properties of WP hydrogel formation induced by different salt ions were observed, and divalent salts were recommended for the formation of more structurally stable WP hydrogels.

4.2. Chemical methods

Chemical methods refer to the formation of intramolecular and intermolecular covalent bonds between monomers or

polymers through the introduction of chemicals, resulting in a 3D network structure. Milk proteins contain a large number of reactive groups, such as amino and carboxyl groups, that are readily modifiable. The obtained milk protein derivatives can be further polymerised or chemically reacted with other reactive groups to obtain chemically constructed milk protein-based hydrogels. These hydrogels typically have higher mechanical strength and stability and can be maintained for a longer period than physically crosslinked milk protein-based hydrogels.[128] Chemical construction methods for milk protein-based hydrogels can be categorised into direct crosslinking and post-modification crosslinking (**Figure 3B**).

4.2.1. Direct crosslinking

Direct crosslinking is mainly performed by introducing other macromolecules to make milk proteins self-crosslinking without the need to modify the milk protein molecules. These macromolecular crosslinkers can act as bridging agents for connecting discrete protein molecules. Milk protein hydrogels obtained by direct crosslinking have higher hardness compared to conventional physical methods.[129] In this section, both casein and WP hydrogels are obtained by inter/intramolecular amide bond formation. Similar to casein, WP does not require preheating treatment to achieve inter/intramolecular crosslinking of proteins. However, the direct crosslinking method tends to take longer due to the stable spherical structure of WP, and preheating treatment can significantly reduce the crosslinking time in WP hydrogel formation.[130,131]

Commonly used macromolecular crosslinkers for milk proteins include glutaraldehyde, formaldehyde, tetra phosphonium chloride, hyaluronic acid, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC)/N-hydroxy succinimide (NHS), genipin, and citric acid. Among them, glutaraldehyde and formaldehyde have been widely used as two of the most effective crosslinkers for crosslinking proteins. Tetra phosphonium chloride reacts with water to produce formaldehyde.[132] Similarly, hyaluronic acid hydrogels require treatment with sodium periodate, which promotes oxidation of hyaluronic acid to produce free aldehyde groups.[133] However, the aldehyde groups of these agents are toxic to cells and can also contribute to environmental pollution. This section now focuses on more promising macromolecular self-crosslinkers, including EDC/NHS, genipin, and citric acid (**Figure 3B1**).

EDC can be defined as a zero distance crosslinker by promoting the formation of protein-amide bonds that ultimately result in a protein conjugate product that is not part of the final crosslinked product and from which all residues can be removed. When used as a crosslinker, it is often combined with its enhancer, NHS, which is added to make the crosslinking more efficient by minimising any rearrangement of the reaction intermediates.[134] Tang *et al.*[87] applied the crosslinking reaction of EDC/NHS for the fabrication of pure BSA protein gels without any synthetic additives and polymers. The results showed that the hydrogel was characterised by high stability, high mechanical properties (compressive/tensile strength of 115/0.43 MPa), low cytotoxicity, and low erythrocyte haemolysis. However, the safety of EDC/NHS is still controversial, although some documents have been published on the production of edible sausages, nanoparticles, etc., treated by EDC/NHS crosslinking.[135,136]

Genipin, present in traditional Chinese medicine and extracted from Gardenia jasminoides, is a natural crosslinker.[137] Its multiple reactive groups, such as hydroxyl groups, react with molecules containing amino groups to form monomolecular or multimolecular crosslinked structures, forming inter- and intramolecular Schiff base bonds.[138] Song *et al.*[88] investigated the factors affecting the crosslinking time of casein hydrogels prepared with genipin. The results showed that the amount of genipin was one of the influencing factors. The crosslinking time could be shortened to 1/6 as the amount of genipin increased from 2.5 mM to 10.0 mM at 35°C. The reaction temperature was another influencing factor. When the genipin concentration was kept at 5.0 mM, the crosslinking time could be reduced by nearly half when the reaction temperature was increased from 35°C to 50°C. However, genipin is more expensive, less efficient at crosslinking, and has a certain degree of cytotoxicity,[139] which may limit its use in milk protein hydrogels.

Citric acid is a readily available, generally recognised as safe, and inexpensive crosslinker. As a poly-carboxylic acid, citric acid can be used to crosslink proteins by forming new intramolecular or intermolecular amide bonds via nucleophilic substitution reactions between the carboxyl group and the protein amino group. Wang *et al.*[89] altered the thermally denatured WP fibres by adjusting the pH to enhance their exposed amino groups and gelation with citric acid. The authors found that the crosslinking mechanisms of preheated WP fibres with citric acid differed at different pH: In addition to amide bonding, the interaction between protonated WP fibres and protonated citric acid promoted the formation of hydrogen bonds under acidic conditions. In contrast, under non-acidic conditions, citric acid-induced deprotonation of WP molecules disrupted hydrogen bonding and promoted amide and disulfide bonding, yielding WP hydrogels with higher modulus. Interestingly, a high concentration of citric acid (400 mM) was unfavourable for WP crosslinking. This is because a high concentration of citric acid reduces the flexibility of the WP fibres, with less inter-fibre space and reduced hydration, which leads to the inability to gel.

4.2.2. Crosslinking after modification

Milk proteins could also be modified to meet specific crosslinking conditions to achieve crosslinking. Among the various construction methods after modification, photoinitiated crosslinking of hydrogels using free radical polymerisation has attracted attention due to their easily controllable parameters (e.g., light intensity, exposure time, or irradiation distance) and in-situ synthesis capabilities.[140,141] In this process, photocleavage generates highly reactive free radicals, which are covalently crosslinked with intra- and inter-molecular groups142 (**Figure 3B2**).

Methacrylates and their derivatives have been extensively manipulated to modify milk proteins to achieve photo crosslinking. In the presence of ultraviolet (UV) light and

photo initiators, the methacryloyl side chains produce free radicals which rapidly crosslink with each other.[143] The degree of crosslinking depends on the number of methacryloyl substitutions. By substituting (95% substitution) the free amino groups on WP with methacrylic anhydride (MA), our team transparent WP-MA hydrogels in the presence of a photo initiator.[39] Wang *et al.*[90] also produced photo crosslinked casein-MA hydrogels by this principle. It is worth noting that the grafting of MA molecules increases the potential risks associated with this hydrogel and may limit its application.

Notably, since tyrosine is a reactive amino acid with a phenolic ring, the phenolic side chains in the tyrosine residues can be crosslinked to each other by forming di-tyrosine bonds through the photoreaction to form a hydrogel without modification. Based on the fact that tyrosine content in casein is high, our team prepared photo crosslinked casein hydrogels using $Ru(bpy)Cl_2$ as a photo initiator, and $(NH_4)_2S_2O_8$ as a cofactor.[41] In addition, we further noted that the reaction system required only white light irradiation to form the gels, which could effectively improve convenience. In contrast, for globular WP (low content and unexposed tyrosine), the mechanical strength of the hydrogel formed is poor, and heat, salt, urea, or acid induction is often required to increase tyrosine accessibility. This was demonstrated in the results of Haas *et al.*[91] By adding 4 M of urea, the G' of the BSA hydrogels was increased by 650%, while simultaneously being more elastic.

4.3. Biological methods

In recent years, the biological construction methods of protein hydrogels dominated by enzyme-induced crosslinking have attracted considerable interest. Enzyme treatment offers three advantages: First, the crosslinkers employed are naturally cell-produced enzymes, rather than harmful chemicals. Second, the gel formation process takes place under mild conditions and is free from the addition of any harmful organic solvents. Finally, the obtained hydrogels are biodegradable, and their products (amino acids and peptides) are nontoxic, reducing possible adverse reactions or complications when used in various biomedical and biotechnological applications.[92,144,145] Similarly, in enzyme-induced crosslinking, WP is not susceptible to enzymes as its globular structure is stabilised by disulfide bridges, and thus pretreatment is needed to expose the corresponding amino acid residues of WP prior to enzyme catalysis.[93,146,147] However, casein does not require these pretreatments due to its natural micellar structure. **Figure 3C** demonstrates the mechanism of the enzyme construction methods of milk protein-based hydrogels.

4.3.1. Transferase crosslinking

Glutamine transaminase (TG) is a transferase widely used for milk protein crosslinking. This enzyme acts in wide ranges of pH (5–8) and temperature (40–70°C) and its activation does not require specific cofactors.[148] TG catalyses the acyl transfer reaction between the γ-formamide (acyl donor) of the glutamine residue and the ε-amino group (acyl acceptor) of the lysine residue to form the ε-(γ-glutamyl)-lysine peptide bond[149] (**Figure 3C1**). In milk protein, more than half of the functional groups are hydrophilic, including exposed glutamine and lysine residues, which makes either casein or WP an ideal target protein for TG.[150] During the process of TG crosslinking, controlling the enzyme activity (pre-incubation, pH and temperature) and the degree of unfolding of the protein fibres can be effective in improving the mechanical strength of the milk protein-based hydrogel.[94] However, Yin *et al.*[151] noted that simple TG crosslinked milk protein-based hydrogels were not sufficiently stable in aqueous solution. Considering the thickening and gelation properties of polysaccharides, they employed konjac glucomannan as the stabilizer for TG-induced casein hydrogels in their experiments. The results showed that the addition of konjac glucomannan significantly improved the stability of the hydrogels, and the degradation rate decreased from 100% to less than 60%. In addition, konjac glucomannan treatment also limited the chain mobility and decreased the swelling rate of the hydrogel. It can be seen that polysaccharides can effectively improve the stability of TG crosslinked milk protein-based hydrogels.

4.3.2. Oxidase crosslinking

In addition to TG, laccase, and tyrosinase have also been used to induce milk protein crosslinking.[98,151] Laccase and tyrosinase both belong to the group of copper-containing polyphenol oxidases that induce crosslinking reactions through an oxidative process, but they differ in their induced principles. As shown in **Figure 3C2**, laccase-induced crosslinking is through the formation of free radicals, while tyrosinase-induced crosslinking is through the formation of quinone intermediates.[95,152]

Laccase has a wide range of substrate specificity and is capable of oxidising a variety of phenolic compounds, amines, etc., with an optimal pH range of 4–5.[153] It oxidises substrates by removing an electron, resulting in the formation of free radicals, which can undergo further polymerisation and hydration, eventually forming covalent bonds.[154] During the process of milk protein crosslinking, covalent bonds catalysed by laccase include disulfide bonds formed between cysteines (predominantly formed in WP hydrogels), di-tyrosine bonds formed between tyrosines (predominantly formed in casein hydrogels), etc.[155] However, it has been noted that the phenolic groups in proteins do not appear to be good substrates in laccase-catalysed reactions.[156] Small phenolic compounds can act as electron transfer mediators in heterogeneous polymer structures acting as bridging agents to facilitate laccase-catalysed crosslinking of proteins. Phenolics with low molecular weight, such as ferulic, caffeic, and chlorogenic acids, enhance the laccase-mediated polymerisation of casein and WP.[95,96] For example, Jiang *et al.*[97] explored the effect of laccase treatment on the physicochemical properties, gel properties, and antioxidant activity of α-LA in the presence and absence of ferulic acid. They found that the gel strength and water retention capacity of α-LA hydrogels treated with laccase and ferulic acid were enhanced and positively correlated with the reaction time.

In contrast to laccase, tyrosinase has the unique dual catalytic function of catalysing both the oxidation of monophenols to o-diphenols and the oxidation of o-diphenols to quinones.[157] The quinone can be further reacted non-enzymatically to form

a polymer. Tyrosine side chains in milk proteins can be oxidised by tyrosinase. It has been reported that tyrosine residues oxidised by tyrosinase can in turn further react chemically with different amino acid side chains present in proteins, such as sulfhydryl, amine, amide, indole, and other tyrosine side chains, forming new covalent bonds that ultimately lead to protein crosslinking.[152] The presence of tyrosine is essential for tyrosinase catalysis. It has been noted that, unlike casein, β-LG and lysozyme in milk proteins are unable to crosslink directly in the presence of tyrosinase due to tyrosine deficiency.[99,151] However, Thalmann and Lötzbeyer99 proposed certain tyrosine-containing substances, such as caffeic acid, could act as a bridging agent in the crosslinking process and facilitate the crosslinking of β-LG and lysozyme. This finding broadened the application of tyrosinase in the crosslinking of milk proteins.

However, enzyme-induced hydrogels have been reported to have some disadvantages, such as having a lower breaking force and therefore being more susceptible to disruption. Yan *et al.*[158] found that enzyme-induced WP hydrogels exhibited higher stiffness and solubility but were poorer in freeze-thaw stability compared to heat-induced WP hydrogels. To overcome this deficiency, Zhao *et al.*[159] explored the effect of ultrasonication on enzyme-induced WP and found that it promoted the binding of the enzyme to enzyme-targeted sites within the WP molecules, increased the degree of crosslinking of the hydrogels, and improved the freeze-thaw stability.

4.4. Interpenetrating polymer network

The interpenetrating polymer network (IPN) is a hybrid system consisting of two polymers that are interwoven in a network, in which at least one of the polymers is crosslinked. The IPN technology can be considered a special type of double crosslinking, and the resulting gels typically have more complex network structures and better mechanical properties, while maintaining the individual properties of each polymer.[160] **Figure 3D** shows a schematic illustration of the synthesis route and linkages of milk protein/other polymer-based IPN hydrogels.

Therefore, it is possible to select polymers with specific properties to compensate for the limitations of milk proteins in terms of reactivity and processability and to expand the functionality and range of applications of milk protein-based hydrogels. Xu *et al.*[100] prepared casein-polyacrylamide (PA) hydrogels by interpenetrating two networks consisting of covalently crosslinked PA and physically crosslinked casein. In this case, casein was induced to form a crosslinked network by acid. PA was induced to form a crosslinked network by free radicals. In the presence of ammonium persulphate (a free radical source) and tetramethylethylenediamine (a free radical polymerisation catalyst), PA hydrogels can be formed by crosslinking of acrylamide monomers and bis-acrylamide.[161,162] Surprisingly, owing to the formation of the dual network, the casein-PA hydrogels exhibited sufficient adhesion and mechanical strength with a maximum peeling force of 378 N/m and a fracture stress of 180 kPa. In addition, the formation of casein-PA hydrogels by photo crosslinking reactions has also been reported.[101] Yet, a careful comparison revealed no significant difference in the composition of the two reaction systems, with the latter only increasing UV irradiation. This could shorten the gel formation time of this IPN hydrogel (2 hours, without UV irradiation - 4 hours), which may be related to the UV-promoted formation of free radicals. However, it has been shown that there is precursor toxicity in PA crosslinking, which limits the use of this IPN in the cell engineering sector.[163]

4.5. Characterisation methods

Since the *in vivo* performance of hydrogels is largely dependent on their properties, it is crucial to identify the basic characteristics of hydrogels before exploring their biomedical potential. The major properties that need to be characterised for a hydrogel include its size and shape, porosity, molecular structures, mechanical properties, hydration properties, biocompatibility, and biodegradability.

The morphology of milk protein-based hydrogels can be analysed using scanning electron microscopy (SEM), cryo-SEM, and transmission electron microscopy. SEM and cryo-SEM provide 3D visualisation of complex structures, and researchers have investigated the fibrillar nature and pore structure of hydrogels using SEM and cryo-SEM, respectively.[164] Transmission electron microscopy has been used primarily to evaluate two-dimensional structural properties of samples, such as length and diameter. For example, Pimont-Farge and colleagues[165] used transmission electron microscopy to observe that increasing the pH of the solution from 9.0 to 11.0 promoted an increase in the number of nano-fibres, which led to a denser network structure in the β-LG hydrogels. The structural changes of the milk protein molecules after crosslinking can be determined using Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. Zhu *et al.*[41] showed that the increase in peak intensity at 1136 cm^{-1} for the photo crosslinked casein hydrogel was due to the stretching vibration of the di-tyrosine C-C bond. Nuclear magnetic resonance spectroscopy similarly confirmed the formation of the di-tyrosine C-C bond. In addition to the previously mentioned techniques, it is crucial to measure the mechanical properties of hydrogels since they can affect cell behaviour. Rheology measures the shear modulus (G) of softer materials, while compression tensile testing measures the Young's modulus (E) of stronger-harder materials. These techniques provide a comprehensive understanding of the mechanical properties of hydrogels.[166] In terms of hydration properties, the swelling ratios reflect the water absorption capacities of the hydrogels. The degradation ratios reflect the ability of hydrogels to maintain structural integrity and sustain mechanical support during the incubation cycle. Last but not least, material safety is usually evaluated by cellular assays for the determination of biocompatibility and biodegradability. Hu *et al.*[39] studied the WP-MA hydrogel and found that the hydrogel had excellent biocompatibility and increased the cellular activity of endothelial cells because of the nutrients of WP. After 4 weeks of implantation, complete degradation of the WP-MA hydrogel occurred. It is not only important to determine the initial properties of the hydrogel, but also equally important to consider how these properties change over time while the hydrogel is still in use. These

properties and their dynamics affect the spatial and temporal behaviour of the hydrogel, thereby controlling its utility in biomedical applications.

In summary, the milk protein-based hydrogels constructed based on physical methods have physical stability and good biocompatibility, but considering the negative effects of denaturation and excessive aggregation, they are usually used as an auxiliary approach for the preparation of milk protein-based hydrogels. In addition, although the milk protein-based hydrogels produced using chemical methods have good stability and high mechanical strength, most of these methods have some toxicity and are not cost-effective. On the other hand, the milk protein-based hydrogels created through biological methods are relatively safe with the advantages of lacking a crosslinker, non-toxicity and mild conditions. However, their weak mechanical strength and their susceptibility to various environmental factors during the preparation process limit their application. It can be concluded that hydrogels constructed by different methods all have corresponding shortcomings. To overcome these limitations and to expand the range of applications, the use of a combination of physical, chemical and biological treatments for the construction of milk protein-based hydrogels may be a good option. The aim of this approach is to utilise the strengths of each method while mitigating their respective deficiencies.

5. Biomedical applications of milk-based hydrogels

According to the application requirements, researchers have continuously adjusted and optimised the properties of milk protein-based hydrogels by chemically modifying milk proteins, introducing reactive units or different construction methods, thereby continuously expanding the depth and breadth of their applications. With excellent properties of biocompatibility, biodegradability, and modification flexibility, milk protein-based hydrogels have broad application prospects in the biomedical field (**Figure 4**). For example, as controlled delivery systems, they can be used as suitable carriers for therapeutic components, improving the flexibility of controlled release, increasing the utilisation of therapeutic component, and preventing adverse reactions. In addition, milk protein-based hydrogels are ideal materials for regenerative medicine, which promote tissue bonding and allow for effective tissue healing and wound repair.

5.1. Controlled delivery

Controlled delivery is designed to prevent degradation of sensitive compounds in response to processing operations, digestive enzymes, and unfavourable environmental conditions (e.g., oxidation, temperature, pH, and light), as well as for site-specific gastrointestinal delivery and controlled release of hydrophilic and hydrophobic nutraceuticals.[167,168] Milk protein-based hydrogels are popular in different industries due to their excellent properties, such as high nutritional value, excellent functional properties, amphiphilicity, biocompatibility, biodegradability, and lower toxicity compared to synthetic polymers.[55,169] Milk protein-based hydrogels can control the release rate of the therapeutic components by forming a variety of strong or weak interactions with the therapeutic components, including hydrophobic interactions, electrostatic interactions, etc., or by controlling the degree of crosslinking and degradation rate. In addition, milk protein-based hydrogels are pH-responsive, allowing for controlled release of the therapeutic components. **Table 3** summarises milk protein-based hydrogels for delivery of therapeutic components.[40,90,125,130,132,170-183]

5.1.1. Inorganic minerals

WP hydrogels are considered to be good carriers of inorganic minerals based on the construction method of salt-induced crosslinking. In salt-induced crosslinking systems, the WP hydrogels can be used to capture and protect micronutrients within their network. In addition to the commonly used calcium and sodium salts, other dietary elemental salts can also induce hydrogels of milk proteins and improve the nutritional properties of hydrogels.

Given the report that the presence of amino acids improves iron bioavailability,[184] the researchers used $FeSO_4$ as the salt-inducing crosslinker to prepare WP hydrogels as a delivery system for Fe^{2+}. Kazemi-Taskooh and Varidi[170] designed a

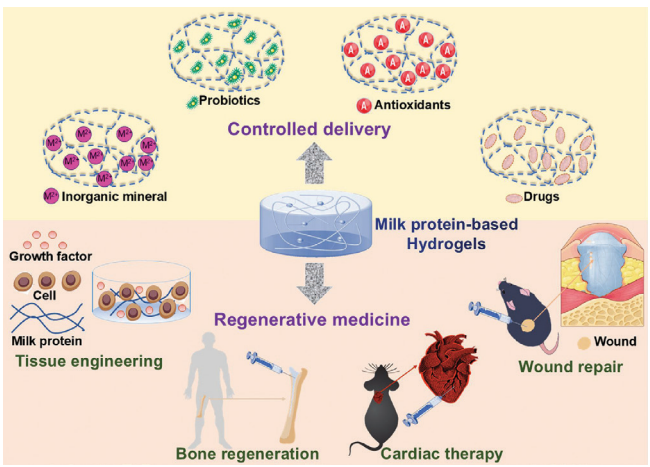

Figure 4. Scheme of the applications of milk protein-based hydrogels in the biomedical fields. Created with Procreate 5.3.13 and PowerPoint 2021.

Table 3. Milk protein-based hydrogels for delivery of therapeutic components

Hydrogel	Gel component	Therapeutic component	Application	Reference
Inorganic mineral	WP/ascorbic acid	Fe^{2+}	Delay Fe^{2+} oxidation rate and increase its bio-accessibility	125
	WP	Fe^{2+}	Transport Fe^{2+} and promote its absorption	40
	WP/gellan gum	Fe^{2+}	Increase the release rate of Fe^{2+} from the hydrogel	170
	β-LG	Fe^{2+}	Improve the bioavailability of Fe^{2+}	171
	WP	Fe^{2+}	Increase the amount of Fe^{2+} captured by WP hydrogels	172
Probiotic	WP/tragacanth gum	*Lactobacillus bulgaricus, Lactobacillus paracasei*	Improve survivability in thermal conditions and stability in the gastrointestinal tract	173
	WP/amylopectin	*Lactobacillus plantarum*	Improve probiotic activity in freeze-drying and storage	174
	WP/amylopectin	*Lactobacillus rhamnosus*	Improve probiotic activity in gastrointestinal	175
	WP/sodium tripolyphosphate	*Lactobacillus plantarum*	pH-responsive gels, potential candidates for gut-specific release of actives	176
Antioxidant	Casein	*Jaboticaba* fruit extracts	Improve anthocyanin stability in the intestine	177
	Casein/pectin	Olive leaf extracts	Improve antioxidant activity of olive leaf extract over a period of 30 days	178
	WP/xanthan gum	Black carrot concentrates	Improve black carrot concentrates stability in simulated gastric fluid	179
Drug	WP	Vitamin B_6	Derive equations for release rate and crosslinking rate	130
	Casein/sodium alginate	Metformin hydrochloride	Improve ability to control releases	90
	WP/chitosan	Riboflavin	Improve curcumin stability in the gastrointestinal tract	132
	WP	Caffeine	Control release of caffeine	180
	WP/sodium alginate	Urea	Retard release in the soil	181
	Casein/chondroitin sulphate	Levodopa	Control drug release to minimize side effects	182
	β-LG/BSA	Riboflavin	Control riboflavin release through amyloidogenic fibre structure	183

Abbreviations: BSA: Bovine serum albumin; WP: Whey proteins; β-LG: Beta-lactoglobulin.

composite hydrogel with WP and gellan gum as an iron carrier to increase the release rate of Fe^{2+} from the hydrogel, and due to the porous structure, the cumulative release amount of iron from this composite hydrogel was increased to 71.74%, and 9.14% in acidic and neutral pH, and iron was released up to 88.55% and 28.72% in simulated gastric and intestinal media. Martin and de Jong[125] optimised the bio-accessibility of Fe^{2+} in the intestine by adding ascorbic acid to iron-containing WP hydrogels to retard the oxidation of Fe^{2+} in the hydrogels, thereby increasing the *in vitro* bio-accessibility of Fe^{2+} from 10% to nearly 80%. Others, Remondetto *et al.*[40] explored the effect of pH on the ability of WP hydrogels to transport Fe^{2+}. They found that WP hydrogels could limit Fe^{2+} release more effectively when pH > pI, compared to pH < pI. In addition, Caco-2 cells could take up a higher amount of iron from WP hydrogels (pH > pI). This phenomenon can be explained by the previously described mechanism of gel formation: Granular hydrogels (pH < pI) have an increased release of Fe^{2+} due to electrostatic repulsive forces between positive charges on the polypeptide chains (protonation of proteins) and the positively charged ferrous ions. In contrast, filamentous hydrogels (pH > pI) can strongly interact with ferrous ions due to the negative charge on the polypeptide chains (protein release of protons), limiting their release. It is evident that filamentous hydrogels are excellent matrices for transporting iron and promoting its uptake.

However, the amount of iron carried by the WP hydrogels is limited. The presence of more randomly aggregated particles at high iron/protein ratios reduces the elastic behaviour and fracture strength of hydrogels as well as limits their ability to transport iron.[171] It was shown that by directing the preheat treatment conditions, the amount of iron captured by WP can be increased. Martin and de Jong[172] investigated the effect of heat treatment conditions (light-moderate-heavy) on the iron-induced hydrogels process of WP to optimise the relationship between gel strength and iron concentration. The results showed that for light (85°C, 30 minutes, pH = 7.0), medium (85°C, 3 hours, pH = 3.35) and heavy (80°C, long time, pH = 2.0), the molar binding ratio of iron: protein increased from 5.3: 1, 8.8: 1 to 17: 1, respectively. Different structural entities may be formed as a function of heating conditions and the amount of iron could be increased from light to heavy heat treatment conditions. In practice, we can change the pretreatment conditions for WP solutions according to the amount of inorganic minerals required.

5.1.2. Probiotics

Probiotics can improve human health by maintaining the balance of intestinal flora, lowering serum cholesterol levels, and regulating immunity, and have wider applications in the pharmaceutical field.[185] To prevent and manage gastrointestinal diseases, they must survive gastric acid and reach at least

1 × 106 colony forming units (CFU)/g in the small intestine.[186] To improve the stability of probiotics reaching the intestine during processing, storage, and oral administration, the use of hydrogel-embedded probiotics is a suitable option. In milk protein-based hydrogels, researchers usually employ WP hydrogels as carriers for loading probiotics based on the fact that WP can promote the production of exopolysaccharides by probiotics.[173]

Protection during processing: Probiotics are susceptible to unfavourable conditions in processing and storage, such as heat, pressure, and oxygen. Sun *et al.*[174] prepared WP concentrate with amylopectin as the hydrogel material for encapsulating Lactobacillus plantarum. The results showed that the survival rate of Lactobacillus plantarum after freeze-drying by this method was 95.64%, which was significantly higher than that of free Lactobacillus plantarum (82.58%). After 240 days of storage, the number of viable bacteria only decreased by 1.05 log CFU/g, and the viability could reach 89.61%. It can be seen that the WP hydrogels have the effect of protecting the probiotics during freeze-drying and storage, based on their good biocompatibility and structural stability.

Probiotics are also vulnerable to inactivation by gastric acid in the body after oral administration. Zhang *et al.*[175] found that WP-amylopectin hydrogel had low solubility in simulated gastric fluid but high solubility in simulated intestinal fluid, suggesting that the gel could protect Lactobacillus rhamnosus under gastric conditions and release probiotics in the intestines to improve the therapeutic efficacy of orally administered Lactobacillus rhamnosus. Based on the influence of pH on the spatial structure and binding capacity of proteins, the hydrogel prepared at pH = 7.5 had the best protective effect. In addition, based on the pH variability across the gastrointestinal tract, pH-responsive hydrogels gained attention from researchers to enable the hydrogels to target the release of probiotics. Zhang *et al.*[176] grafted pH-responsive motifs (-OPO^{3-}) through the reaction of lysine residues of WP with sodium tripolyphosphate (STP), resulting in the phosphorylation of the lysine residues. The results revealed that the swelling behaviour of the modified WP hydrogels was sensitive to the environmental pH. In an acidic system, the water molecules entry was inhibited by the contraction of the protein chains. In alkaline environments, acidic protons dissociated from the protein side chains and increased electrostatic repulsion, leading to gel network expansion and facilitating the entry of water molecules. The survival rate of probiotics was increased by 6.41 log after 30 minutes immersion in an acidic environment (pH 2.0) compared to unmodified WP. Therefore, WP-STP hydrogel could be a potential candidate for gut-specific release of active substances. However, the extent of STP grafting was limited, and excessive STP content increased the degree of crosslinking between protein molecules, which decreased the solubilisation rate of WP hydrogels. In another study, Lu *et al.*[187] synthesised a heparin-poloxamer hydrogel for the loading of Lactobacillus lactis to promote angiogenesis. Based on the fact that milk proteins can enhance bacterial activity and promote biomolecule production, we hypothesise that a hydrogel constructed with milk proteins as a carrier for Lactobacillus lactis could exhibit superior performance.

5.1.3. Antioxidants

Natural antioxidants are employed to preserve product quality and prolong their shelf life. However, their direct incorporation into matrices is challenging due to their unfavourable aftertaste, involvement in enzymatic browning reactions, low stability under environmental conditions (light, oxygen, and temperature), and limited bioavailability. Encapsulating antioxidants in milk protein-based hydrogels is a strategy to improve their stability and bio-accessibility.

Unlike embedding probiotics, in milk protein hydrogel-embedded antioxidant applications, antioxidants can produce hydrophobic interactions with proteins, which can increase the transport capacity of the material. Both casein and WP hydrogels are ideal carriers for loading antioxidants. However, WP hydrogels tend to have higher digestibility based on the relatively weaker network structure of WP hydrogels compared to casein hydrogels under the same crosslinking conditions. Nascimento *et al.*[177] found that casein hydrogels could be used to carry and transport Jaboticaba fruit extracts (mainly anthocyanins) and that the pH of the hydrogel had a large effect on the amount of the substance released. As previously mentioned, the maximum release of this delivery system was observed at pH 7.0 due to protein protonation, however, the minimum release was observed at pH 2.0 since anthocyanins form strong hydrophobic interactions with proteins before acidic pH values. Ozel *et al.*[179] explored the effect of WP hydrogels with pectin, yarrow gum, and xanthan gum as additional polymers on black carrot concentrates (similar to anthocyanins). After nuclear magnetic resonance spectroscopy relaxation and Fourier transform infrared spectroscopy measurements, the authors found that the xanthan gum-containing WP hydrogels prolonged the rate of black carrot concentrate release. Thus, the presence of polysaccharides in the hydrogel system can have strong and complex physical interactions with the WP molecules, which can significantly affect the sensitivity of WP hydrogels to protein digestion.[178,188]

5.1.4. Drugs

When taken orally, drug molecules should be selectively accumulated in the diseased area to improve therapeutic efficacy and reduce side effects, but most drug molecules suffer from insufficient selectivity and low bioavailability. Therefore, it generally requires the use of specific drug carriers for targeted delivery.[180,181,189,190]

To achieve this, casein and chondroitin sulphate were combined to form a novel hybrid hydrogel for encapsulating levodopa.[182] This hydrogel had the best results in terms of drug capture and release at 70% casein and 30% chondroitin sulphate (t_{50} = 13 hours and t_{70}= 87 hours), and the release profiles were in good agreement with the model of Korsmeyer-Peppas and Weibull. It has also demonstrated excellent compatibility, without cytotoxicity, and served as a good scaffold for drug delivery. In addition, the amyloid milk protein also affects drug release. How *et al.*[183] investigated the control effect of β-LG/BSA

hydrogels containing amyloidogenic fibrils on riboflavin. The results of hydrogel characterisation showed that the number of β-LG amyloid protofibrils determined the mechanical properties and surface microstructure of the β-LG/BSA amyloid-based hydrogels. The incorporation of BSA promoted the formation of β-LG protofibrils, preventing conformational change of β-LG protofibrils at elevated pH, and modulated the hydrogel by reducing the entanglement of protofibril chains and increasing the surface homogeneity of the hydrogel. The mechanical properties of the hydrogels were adjusted by reducing the entanglement of protofibril chains and improving the surface homogeneity. The results of controlled drug release showed that the riboflavin release rate was correlated with the number of β-LG amyloid protofibrils and the binding affinity between BSA and riboflavin, and the release rate was mainly depended on the number of amyloid protofibrils, which could be adjusted by the amount of BSA. Therefore, BSA plays an important role in regulating the properties of β-LG amyloid hydrogels and the slow release of riboflavin.

5.2. Regenerative medicine

In recent years, regenerative medicine has played an increasingly important role in the repair and regeneration of defective tissues, in which scaffolding materials are one of the key factors influencing the growth, proliferation, and differentiation of stem cells.[191] Based on their low cost, excellent biocompatibility, and biodegradability, natural milk protein-based hydrogels are considered ideal scaffold materials for applications in regenerative medicine.

5.2.1. Tissue engineering

Cells show considerable promise for a variety of preclinical and clinical applications, such as drug screening and disease treatment.[192] These applications require the production of large quantities of high-quality cells. In tissue engineering, cells serve as the "seeds" that are cultured on scaffolds and then proliferate to large-scale *in vitro*. Conventional two-dimensional culture platforms for large-scale cell expansion are usually inefficient, often resulting in altered cell phenotypes, limited expansion, and senescence after multiple passages.[193] The survival, proliferation, migration, and other functions of cells are widely dependent on the interactions with intercellular and extracellular matrix, which require a 3D environment. 3D culture methods, especially hydrogel culture methods, have the potential to improve cell expansion.[194] Several hydrogel-based systems have been developed for cell culture and *in vitro* expansion. Milk proteins, especially WP, have been reported to promote the growth and differentiation of stem cells.[195] Therefore, WP hydrogels offer unique advantages in cell culture application of tissue engineering.

For hydrogel scaffolds used in cell culture, cell adhesion is also considered essential.196 Based on the fact that LF contains naturally occurring arginine-glycine-aspartic acid (RGD) peptides, Reyhani *et al.*[197] mixed LF with sodium alginate to form a gel for culturing MG-63, an osteoblast cell line. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cell viability showed that LF-sodium alginate hydrogels were successful in promoting the adhesion and growth of MG-63. However, with the exception of LF, none of the other milk proteins have functional groups (e.g., RGD sites) for cell adhesion, which limits the application of milk protein-based hydrogels in cell culture. Researchers are exploring the possibility of conferring cell adhesion to milk protein hydrogels without RGD peptides, either through chemical or physical modification. Charron *et al.*[54] showed that the WP-MA hydrogel formed by chemical modification of WP (grafting MA) could successfully promote the adhesion of myoblasts, which further proliferated and differentiated into myotubes. The formation of double bonds by grafting MA increases the surface energy of the material. When the polymer surface interacts with biological fluids, serum protein adsorption and cell adhesion depend on the surface energy of the polymer. A surface with high free energy favours cell adhesion and spreading, while low free energy surfaces inhibit cell behaviour.[198] Alternatively, air plasma treatment can ionize atmospheric gases such as oxygen, hydrogen, or nitrogen through an electromagnetic field to form a non-thermal plasma. These charged substances can introduce groups such as carbonyl, carboxyl, and hydroxyl groups to the surface of the polymeric material, which enhances their binding with extracellular matrix or cell surface proteins.[199] Ong *et al.*[200] prepared stabilised, semi-transparent BSA hydrogels by controlled heat-induced crosslinking and the addition of sodium chloride. The resulting biologically inert hydrogels were then subjected to air plasma treatment, functionalising the surface to enable attachment of a basement membrane matrix (Geltrex). Compared to untreated samples, the functionalised BSA hydrogels showed good cell adhesion, foetal human osteoblasts could proliferate stably on the material, and the gene expression profile of osteoblasts was preserved and higher.

5.2.2. Bone regeneration

Bone can repair small cracks and fractures on its own. However, when the bone defect exceeds 2 cm, the bone's natural repair mechanisms are insufficient.[201] While traditional repair methods for bone injury, such as autografts, allografts, and xenografts, have been widely utilised, they all have corresponding drawbacks, limiting their clinical application. Despite the development of a variety of biomaterials, including metal implants, calcium phosphate cement, hydroxyapatite, etc., they have not yet fully achieved the desired therapeutic effect.[202] Currently, polymer scaffolds, especially hydrogels, are of interest and have been extensively studied for their unique configuration and tuneable physicochemical properties.[203,204]

Based on the fact that WP has been shown to act as an enhancer of osteogenic differentiation, the use of WP hydrogels as a biomaterial for bone regeneration appears to be a promising approach compared to casein hydrogels, especially due to the low cost of WP.[195] Gkioni *et al.*[205] employed a biomimetic, marine-inspired approach to mineralise WP hydrogels with an inorganic phase consisting of $CaCO_3$ (mainly calcite) and $CaCO_3$ enriched with magnesium using the calcifying enzyme urease. The results showed that mineralised and unmineralised hydrogels were non-cytotoxic and promoted cell viability to comparable extents (approximately 74% of standard tissue culture polystyrene). WP hydrogels, both in their unmineralised and mineralised forms (with $CaCO_3$ and magnesium-enriched

$CaCO_3$), show potential as effective biomaterials for bone regeneration. This WP hydrogel was formed by a simple heat-induced crosslinking method, and the high mechanical strength obtained was favourable for improving osteoblast activity. In the field of bone regeneration, the incorporation of bioactive inorganic phases (e.g., $CaCO_3$,[206] CaO, and SiO_2)[207] into the WP hydrogel matrix provides the hydrogel with a mineralisation capacity, ensuring direct chemical bonding of the hydrogel to the bone and further increasing the incorporation of the material in the surrounding bone tissue. Furthermore, in terms of mechanical properties, the incorporation of inorganic mineral phases also improves the mechanical properties of the WP hydrogel, thereby promoting cell adhesion, proliferation, and osteogenic differentiation. However, concluded their study at the cellular testing stage without conducting animal studies for *in vivo* validation, which warrants further investigation.

5.2.3. Cardiac therapy

Regenerative medicine solutions are also of particular interest for the treatment of heart disease, such as myocardial infarction (MI), where substantial functional tissue is lost and intrinsic regenerative capacity is severely limited. MI is caused by temporary or permanent occlusion of the major coronary arteries, resulting in a significant reduction in blood supply to the beating heart muscle in the left ventricle.[208] To compensate for the low and insufficient intrinsic regenerative capacity of the adult heart, therapeutic regenerative strategies with hydrogels have attracted scholarly attention.[209] 3D hydrogels can be implanted *in vivo* to provide mechanical support to failing myocardium, promoting myocardial repair and tissue reconstruction. Milk protein-based hydrogels may be feasible for the treatment of MI due to their safety, biocompatibility, and biodegradability as a potential biomaterial.

To verify the above, our team prepared WP-MA hydrogel.[39] We found that the G' of WP-MA hydrogel was 20 kPa, which was in line with the G' range (3–200 kPa) of hydrogels prepared by predecessors for the treatment of MI.[210,211] We also verified metallopeptidase-9 (MMP-9) cleavage site in the material, which supports the excellent degradability of the material. The left ventricular tissue of the WP-MA hydrogel-treated group was thickened, and the proportion of fibrotic tissue in the left ventricular tissue was significantly reduced, and it was completely degraded at 28 days after injection. These results suggested that WP-MA hydrogel temporarily mechanically supported the maintenance of myocardial function and attenuated adverse left ventricle remodelling after MI. In addition, based on the ability of Cu^{2+} to modulate metabolic homeostasis after MI and the superior ability of milk protein-based hydrogels as controlled delivery, we hypothesised that milk protein-based hydrogels could offer advantages in cardiac therapy as carriers with delivery of Cu^{2+}. Our team verified the Cu^{2+} binding capacity of different protein-based biomaterials and performed molecular docking simulations to screen for those with high Cu^{2+}/protein binding ratios.[212] We evaluated proteins commonly used in tissue engineering, including collagen, filaggrin, BSA, human serum albumin and casein. The results showed that casein exhibited the most superior Cu^{2+} binding capacity, more than twice that of the other proteins. The casein hydrogels containing Cu^{2+} (named as CuCMG) prepared by our team were found to significantly attenuate MI-induced cardiac dysfunction and maladaptive remodelling with increased angiogenesis.

The flexibility in processing allows the photo-crosslinked milk protein-based hydrogel to be adapted into different forms, including particles and customised scaffolds, rendering its adaptability with different implantation methods, including minimally invasive injection. In this section, milk protein-based hydrogels are not expected to support rapid tissue integration in the same way as gelatine-based hydrogels derived from cell adhesion.[213] However, the non-adhesive nature of our prepared hydrogels may contribute to their use as anti-adhesion barrier materials. We suggest that in milk protein-based hydrogels, the active peptides can exert their bioactive function via two similar routes: direct contact with the surrounding and recruited cells or interaction with cells from inside. As suggested by their principles, the effect of direct contact is expected to be activated immediately, while hydrogel degradation-dependent interactions are expected to be initiated significantly later.

5.2.4. Wound repair

Milk protein-based hydrogels have good biocompatibility, degradability, and the ability to load and prove controlled release of a variety of antibacterial and anti-inflammatory drugs, making them suitable for wound repair treatment. The crosslinking duration of the hydrogel needs to be as short as possible due to the continuous bleeding characteristic of wounds. Based on this property, photo-crosslinked milk protein-based hydrogels, which can be synthesised in situ, are ideal candidates for wound dressings.

Our team synthesised casein hydrogels in situ on mouse wounds by a di-tyrosine photo crosslinking mechanism based on tyrosine-rich fragments of casein.[41] The results demonstrated that the casein hydrogel could be formed under endoscopic light in only 2 seconds, which was favourable for its use in emergency and minimally invasive procedures. The photo-crosslinked casein hydrogel was covalently bound to tissues and exhibited strong adhesive properties. It was also able to withstand blood pressures over 180 mmHg and had excellent haemostatic properties. We further found that casein hydrogel promoted wound healing by decreasing the levels of inflammatory factors such as tumour necrosis factor-alpha, interleukin-1beta, and interleukin-6 and upregulating the levels of transforming growth factor-beta, which promotes cell proliferation and differentiation. The percentage of wound closure for casein hydrogel bio-adhesive treatment was approximately 53.5% after 4 days, whereas the percentage of wound closure for blank and commercially available fibrin gel treatments was lower (28.6% and 46.4%, respectively). Based on these results, photo-crosslinked casein hydrogel could be used as a novel wound dressing to rapidly stop bleeding and promote post-traumatic wound healing. In another study, our team also demonstrated the use of tryptophan-rich α-LA to produce a novel natural protein hydrogel suitable for wound dressing.[42] To create photo crosslinkable polymers, the authors subjected α-LA to methacrylate. The obtained α-LA-MA hydrogel showed good biocompatibility and degradability *in vivo* tests

and could accelerate post-traumatic wound healing. The wound healing rate for the α-LA-MA hydrogel bio-adhesive treatment was approximately 75% after 4 days, compared to only 60% and 68% for the blank and gauze treatments. In addition, only the wounds of mice treated with α-LA-MA were completely closed and covered with new epidermal tissue 10 days after incision. This may be because the high presence of tryptophan promotes the synthesis of serotonin (associated with blood coagulation and healing), which accelerates the migration and proliferation of keratinocytes and fibroblasts and promotes the healing of skin wounds.[214]

In this section, based on the high tyrosine content in casein, casein hydrogels can be directly photo-cured for applications in wound treatment. Whereas α-LA hydrogels have the advantage of promoting keratinocyte growth in wounds, based on their high tryptophan content.

5.3. The relevant preclinical animal experiments and an exploratory clinical pilot study

Preclinical animal experiments are an important means of evaluating the safety margins and the therapeutic effect of new materials through the establishment of animal models. In contrast, a clinical pilot study is a requisite initial step in exploring a novel intervention or an innovative application of an intervention. A pilot result can inform feasibility and identify modifications needed in the design of a larger, ensuing hypothesis testing study.[215] **Table 4** shows the current preclinical animal experiments and an exploratory clinical pilot study related to milk protein-based hydrogels.[39,41,42,212,216] Currently, laboratory studies on milk protein-based hydrogels have been quite extensive. However, most of the studies have focused mainly on *in vitro* experiments. At present, some preclinical animal experiments have only confirmed the usefulness of various types of milk protein-based hydrogels in cardiac therapy and wound repair in mice as we mentioned before. In a noteworthy clinical pilot study, Ito *et al.*[216] evaluated the safety and efficacy of the milk protein concentrate hydrogel through epicutaneous immunotherapy involving the skin's immune system. The researchers applied it to the skin of patients with severe milk allergy. The study utilised the immunogenicity of the milk protein concentrate contained in the hydrogel to elicit an immune response by delivering the allergen to Langerhans cells and/or dermal dendritic cells through the intact skin, and a very small amount of milk protein concentrate was delivered to the intact skin. In this pilot study, four of eight subjects had an increased symptom induction threshold that allowed them to drink milk. Yet, a pilot study does not provide a meaningful effect size estimate for planning subsequent trials due to the imprecision inherent in data from small samples.

Despite the absence of current clinical trials. Milk protein-based hydrogels exhibit significant potential for clinical applications due to their unique properties and versatility. Specifically, in the field of controlled drug delivery, these hydrogels can achieve precise release of therapeutic agents by modulating their pore structure and crosslinking density, while their degradation products are non-toxic, ensuring safety for medical use. Furthermore, leveraging the ability of milk proteins to enhance the production of exopolysaccharides by probiotics, these hydrogels can serve as effective carriers for functional foods, facilitating the delivery of probiotics to aid in the management of chronic diseases such as diabetes and inflammatory disorders. In regenerative medicine, the antimicrobial and antioxidant properties of milk proteins and their hydrolysates make milk protein-based hydrogels promising candidates for wound dressings to promote healing. However, despite their promise, the clinical applications of milk protein-based hydrogels indeed face several challenges. Firstly, the low mechanical strength of milk protein hydrogels makes them prone to deformation or rupture under external forces or prolonged use, limiting their suitability for mechanically demanding conditions. Secondly, controlling the stability and degradation rate of hydrogels *in vivo* remains a significant challenge based on their easily degradable nature, which may affect drug release and tissue repair. Lastly, the lack of adhesion sites in milk proteins also increase the technical barrier to clinical applications, while the introduction of modifiers may further increase the complexity of *in vivo* immune responses.

Table 4. The relevant preclinical animal experiments and an exploratory clinical pilot study applying milk protein-based hydrogels

Category	Target	Aim	Hydrogel material	Pathway	Mechanism	Reference
Preclinical animal experiment	Heart	MI therapy	WP-MA	Operative treatment	Temporary mechanical support from the WPI-MA hydrogel maintains the cardiac function and attenuates adverse left ventricular remodelling after MI	39
			CuCMG	Operative treatment	CuCMG rescues myocardial metabolism after MI by sustained release of Cu^{2+} in the myocardium.	212
	Skin	Wound repair	Casein	Surface treatment	Decrease the levels of inflammatory factors such as TNF-α, IL-1β, and IL-6 and upregulate the levels of TGF-β, which promotes cell proliferation and differentiation	41
			α-LA-MA	Surface treatment	The creation of neurotransmitters such as serotonin and its enhancing effect on the barrier function	42
Clinical pilot study	Skin	Allergy therapy	MPC	Surface treatment	Through epicutaneous immunotherapy, the immunogenicity of MPC that penetrates the skin is utilised to elicit an immune response from the body	216

Abbreviations: CuCMG: The casein hydrogels containing Cu^{2}+; IL-1β: Interleukin-1beta; IL-6: Interleukin-6; MA: Methacrylic anhydride; MI: Myocardial infarction; MPC: Milk protein concentrate. TGF-β: Transforming growth factor- beta; TNF-α: Tumour necrosis factor-alpha; WP: Whey proteins; α-LA: Alpha-lactalbumin.

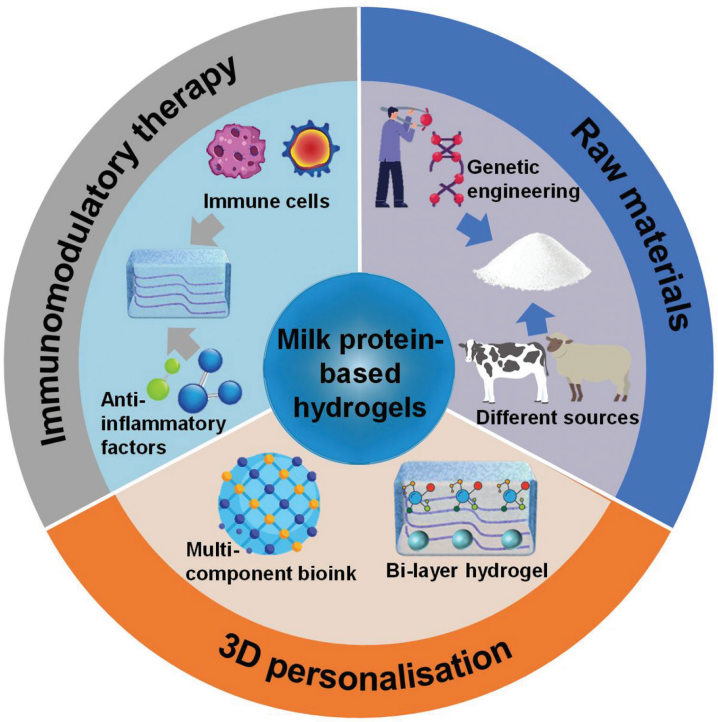

Figure 5. Future directions for milk protein-based hydrogels. Created with Procreate 5.3.13 and PowerPoint 2021. Abbreviation: 3D: Three-dimensional.

In summary, milk protein-based hydrogels presented great potentials as controlled delivery carriers. Utilising their ability to respond to different environmental factors, milk protein-based hydrogels provide an effective means to achieve sustained drug delivery, opening up opportunities to improve therapeutic efficacy. In addition, they can be used in the development of regenerative medicine. The biocompatibility of milk protein-based hydrogels is an ideal property for regenerative medicine, ensuring that humans are not harmed on contact. Over time, milk protein-based hydrogels break down naturally, reducing the need for surgical removal or intervention, which is particularly beneficial for *in vivo* materials used in regenerative medicine applications.

6. Conclusions and prospects

Milk proteins represent an interesting class of polymeric biomaterial due to their low cost, good biocompatibility, degradability, and specific physiological functions. This paper reviews the various construction methods of milk protein-based hydrogels and their representative advances in biomedical applications. Depending on the structures and properties of milk proteins, milk protein-based hydrogels can be constructed by physical, chemical, and biological methods. The selection of suitable preparation methods to control properties such as degradation and strength as well as the possibility of promoting cell growth makes milk protein-based hydrogels ideal candidates for biomedical applications. Milk protein-based hydrogels have been shown to have several advantages, including i) providing good biocompatibility and biodegradability; ii) serving as controlled delivery carriers for therapeutic components and preventing the loss of efficacy of sensitive bioactive substances in different environments; and iii) serving as regenerative medicine materials capable of providing a stable interface for moist biological tissues or organs. Although the development of milk protein-based hydrogel materials is currently gaining momentum, the related research is not yet sufficiently deep and systematic, and there are still some bottlenecks and challenges in the future. **Figure 5** illustrates the novel possibilities for the personalisation of milk protein-based hydrogels.

With the increasing demand for new and personalised biomaterials, genetic engineering provides a new approach to design novel biopolymers to match the specific functions of biomaterials. For example, researchers have introduced genes promoting Ca^{2+} binding into silkworm eggs by microinjection, and silk proteins with strong calcium ion binding ability were obtained through expression, which can be used for the preparation of bone repair materials.[217] In the future, by modifying milk proteins at the molecular level using genetic engineering techniques, we can obtain milk proteins with new functions, thus preparing genetically engineered functional milk protein-based hydrogels to diversify the application scenarios and meet the needs of modern biomedicine. In addition, researchers should conduct systematic differential studies to explore in depth the differences between different sources of milk proteins. This includes, but is not limited to, studies on the composition, structure, and functional activity of different sources of milk proteins to better define the possible biomedical significance of the correspondingly prepared milk protein-based hydrogels.

The application of 3D printing technology can be personalised in an extremely precise and individualised manner to suit the needs of each patient.[218] While the literature has demonstrated the application of photo-crosslinked milk protein-based hydrogels, however, in the case of printing replacement organs consisting of complex and coordinated cellular and extracellular matrix components and microvascular systems,

however, existing 3D printed milk protein-based hydrogels are not sufficient to accurately mimic *in vivo* tissues for the desired function. One of the existing challenges is the mechanical properties of the structures, especially for load-bearing tissues with high natural mechanical strength, such as cartilage and bone. Milk protein-based hydrogels are typically soft due to their highly hydrated nature, resulting in difficulties in achieving high Young's modulus, as seen in natural human articular cartilage. Therefore, a new trend has emerged to develop high-strength multicomponent bioinks to overcome these obstacles. For example, researchers have utilised the reinforcement of gelatine-MA with the addition of poly(N-acryloyl 2-glycine).[219] The double hydrogen bonding of the poly(N-acryloyl 2-glycine) side chains strengthens and stabilises the gelatine-MA network, resulting in structures with outstanding compressive strength (up to 12.4 MPa). In addition to providing suitable post-printed structures, 3D printed milk protein-based hydrogels should also be able to support target cellular functions and facilitate the incorporation of multiple additional components.[220] For example, different tissue types require different cell-directed components to build the appropriate ecological niche microenvironment to support the necessary cell signalling and behaviour. Therefore, the new generation of multi-component milk protein-based bioinks should be able to spatially control the distribution of other components in the printed structure to meet the needs of multiple cell types. Furthermore, combinations with different biopharmaceuticals could better meet site-specific needs. A bilayer porous scaffold constructed with gelatine-MA hydrogel as a matrix includes an upper layer with bioactive peptides that can adsorb transforming growth factor-beta 1 for cartilage repair and a lower layer with hydroxyapatite for subchondral regeneration.[221] Hence, the development of the next generation of milk protein-based bioinks should include these novel bioactive components.

This personalised and customised milk protein-based hydrogel exhibits great promise for immunomodulatory therapy. At the time of tissue injury, proper inflammation is the key event that initiates the process of tissue regeneration. In the early stages of inflammation, immune cells produce cytokines and phagocytose cellular debris and pathogens. Thereafter, anti-inflammatory cytokines are secreted to resolve inflammation and promote tissue regeneration. However, a prolonged inflammatory response can impede wound healing. Based on the fact that inflammatory responses are intimately associated with the overproduction of reactive oxygen species (ROS).[187,222] Peng *et al.*[223] utilised the anti-ROS oxidative damage ability of Cu5.4 Oultrasmall nanoparticles to attenuate ROS production, thereby blocking further inflammatory activation signals in diabetic wounds. The heparin-polyethylene glycol hydrogel, which was used as the carrier, was prepared by covalent crosslinking between the carboxyl group in sodium heparin and the amino group in star polyethylene glycol. We believe that based on the promising antioxidant of milk proteins, it may be possible to further attenuate the generation of ROS at the wounds as well as inhibit the overexpression of MMP if milk proteins are used as the supply material for the amino groups in the gel network.[224] In the future, novel milk protein-based hydrogels could be further explored for encapsulating anti-inflammatory factors cc and immune cells for immunomodulatory therapy.

In conclusion, with the ongoing exploration of the functionalisation and intelligence of milk protein-based hydrogels, their development in the biomedical field will become increasingly sophisticated.

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Conflicts of interest statement
The authors declare that they have no commercial or affiliated interests that represent a conflict of interest in association with this article.

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