Advancements in Gelatin-Based Wound Dressings: Cross-Linking Techniques, Biocompatibility, and Biodegradability for Enhanced Angiogenesis and Healing

Ishita Singha[1], Bani Kumar Jana[1], Niva Rani Gogoi[1], Hemanta Pathak[1], Mohini Singh[1], Bhaskar Mazumder[1*]

Abstract

Uncontrolled hemorrhage from wounds is a significant cause of morbidity, highlighting the need for rapid and effective hemostatic interventions. Wound dressings play a crucial role in managing blood loss and fluid exudation while supporting the dynamic wound-healing process. This process involves hemostasis, inflammation, proliferation, angiogenesis, and remodeling to restore the skin's barrier function. Among available options, dry absorbable local hemostats offer practical advantages by absorbing wound exudates and promoting healing. Gelatin sponges, known for their hemostatic properties, biocompatibility, and biodegradability, are excellent candidates for wound dressings. They effectively stop bleeding and support tissue repair. However, gelatin's inherent sensitivity to environmental conditions limits its direct application. To address these challenges, cross-linking techniques are employed to improve mechanical strength, hydrolysis resistance, and stability while refining the pore morphology of gelatin sponges for enhanced biomedical applications. Various cross-linkers, such as aldehydes, carbodiimides, reducing sugars, genipin, and acyl azides, have been investigated for gelatin modification. While glutaraldehyde achieves effective cross-linking, its high cytotoxicity restricts its use in biocompatible products. Alternatives like 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and genipin offer reduced cytotoxicity, though genipin's cost poses a limitation. Reducing sugars, such as glucose and fructose, provide a promising, non-toxic, and cost-effective solution for commercial-scale production. Optimizing cross-linking methods is essential for developing safe, effective, and affordable gelatin-based wound dressings. Addressing cross-linker toxicity remains critical to advancing their clinical applicability.

Keywords: Gelatin, Hemostats, Cross-linkers, Sponge, Inflammation, Angiogenesis, Wound healing.

Significance | This review discusses the use of gelatin sponges for safer, more effective, and affordable wound dressings for improved clinical outcomes.

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Editor Md Shamsuddin Sultan Khan And accepted by the Editorial Board November 02, 2024 (received for review September 09, 2024)

Introduction

The skin, the largest organ of the human body, serves as the outermost protective layer, forming the primary interface between the body and its environment. It functions as a crucial barrier against foreign pathogens and plays a vital role in maintaining homeostasis (Li et al., 2017; Byrd et al., 2018; Hu et al., 2018; X. Wang et al., 2020). Despite its protective properties, the skin is frequently subjected to damage caused by physical trauma, chemical exposure, or diseases, affecting millions of individuals annually (Hu et al., 2018). Wounds resulting from these factors represent a significant source of morbidity and can lead to severe

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Please Cite This:
Ishita Singha, Bani Kumar Jana, Niva Rani Gogoi, Hemanta Pathak, Mohini Singh, Bhaskar Mazumder (2024). "Advancements in Gelatin-Based Wound Dressings: Cross-Linking Techniques, Biocompatibility, and Biodegradability for Enhanced Angiogenesis and Healing", Journal of Angiotherapy, 8(11),1-13,10011

complications if not managed effectively (Howell-Jones et al., 2005).

Uncontrolled bleeding is a leading cause of death in battlefield scenarios, traffic accidents, and natural disasters. In such emergencies, the inability to quickly stop hemorrhaging often results in increased mortality and complications, particularly before medical care becomes accessible (Fan et al., 2021; Kheirabadi et al., 2010). Open wounds pose an additional risk of infection, which can progress to tissue necrosis and life-threatening conditions if not properly treated (Hu et al., 2018; X. Wang et al., 2020). Thus, rapid bleeding control and effective wound management are critical to preventing infections and restoring skin functionality temporarily.

Wound dressings are a cornerstone in wound care, designed to protect against fluid and protein loss, provide a barrier against infections, and support the healing process. Ideal wound dressings should exhibit properties such as biocompatibility, water absorbency, biodegradability, non-cytotoxicity, and antibacterial activity (Imani et al., 2013; Li et al., 2017; T. Wang et al., 2012; X. Wang et al., 2020). By enabling gaseous exchange and absorbing excessive exudate while maintaining local moisture, wound dressings accelerate healing and support cell proliferation (Hu et al., 2018).

Traditional hemostatic materials such as gauze, cotton, and tourniquets often fail to effectively manage severe bleeding, particularly in cases involving irregular penetrating injuries or hemorrhagic shock. Consequently, the development of fast-acting, safe, and effective hemostatic materials suitable for emergencies and surgical applications is imperative (Fan et al., 2021). Absorbable local hemostats have emerged as a promising solution, capable of stopping bleeding and managing wound exudates. By removing excess exudate, which facilitates wound healing, these materials promote an optimal environment for recovery. Additionally, they offer several advantages, including reduced blood loss, avoidance of systemic hemostatic drug side effects, and reduced reliance on transfused blood (Imani et al., 2013; Tomizawa, 2005).

Dry absorbable hemostats are particularly beneficial due to their capacity to absorb several times their weight in water or bodily fluids, subsequently expanding to fill wound cavities. These materials are tailored to enhance hemostasis and minimize postoperative complications. A wide array of commercially available hemostatic products, including gauze, sheets, powders, foams, and sponges, address varying degrees of bleeding. Among these, foams and sponges stand out as the most convenient, offering ease of application and adaptability to diverse wound sites (de la Torre et al., 2007; Tomizawa, 2005).

Given the limitations of traditional approaches, innovative hemostatic materials with enhanced functionality are essential to improving patient outcomes in emergency and surgical scenarios. These advancements underscore the critical role of local hemostats in modern wound management and their potential to revolutionize care for trauma patients.

2. Pathway of wound healing

Wound healing is a highly organized biological process that occurs in distinct but overlapping stages: hemostasis, inflammation, proliferation, and remodeling. These stages involve intricate interactions among various cells, signaling molecules, and the extracellular matrix to restore tissue integrity and functionality.

2.1 Hemostasis

Hemostasis is the immediate response of the body to prevent blood loss following vascular injury. Damage to arterial vessels triggers rapid constriction due to the contraction of smooth muscle in the circular layer of the vessel wall. This vasoconstriction reduces blood flow, leading to tissue hypoxia and acidosis, which in turn promotes the production of vasoactive metabolites such as adenosine and nitric oxide (NO). These metabolites induce reflex vasodilation and arterial relaxation, while histamine release from mast cells increases vascular permeability, facilitating the migration of inflammatory cells into the extracellular space around the wound (Singh et al., 2017; Young & McNaught, 2011).

Simultaneously, clot formation prevents further blood loss. This process is regulated by the intrinsic and extrinsic coagulation pathways and platelet activation. Platelets not only form the initial clot but also release multiple growth factors and cytokines, such as platelet-derived growth factor (PDGF), which regulate downstream healing processes (Singh et al., 2017; Young & McNaught, 2011).

2.2 Inflammation

The inflammatory phase aims to prevent infection and clear debris. Following injury, the mechanical barrier of the skin is disrupted, leaving the tissue vulnerable to microbial invasion. Neutrophils are the first responders, infiltrating the wound within an hour of injury and continuing their activity for 48 hours. This migration is mediated by chemical signaling mechanisms such as the complement cascade, interleukin activation, and transforming growth factor-beta (TGF-β) signaling. These signals guide neutrophils to the wound site via chemotaxis (Singh et al., 2017; Young & McNaught, 2011).

Neutrophils employ three mechanisms to combat debris and bacteria:

2.2.1 Phagocytosis – Engulfment and destruction of foreign particles.

2.2.2 Degranulation – Release of toxic substances like proteases, lactoferrin, and neutrophil elastase to degrade pathogens and necrotic tissue.

2.2.3 Neutrophil extracellular traps (NETs) – Extracellular chromatin and proteases capture and neutralize bacteria.

Neutrophil activity generates reactive oxygen species, which possess bactericidal properties but may also sterilize the wound via interaction with chlorine. After fulfilling their role, neutrophils undergo apoptosis and are removed by macrophages, which infiltrate the wound 48–72 hours post-injury (Singh et al., 2017; Young & McNaught, 2011).

Macrophages thrive in the acidic wound environment and release growth factors such as TGF-β and epidermal growth factor (EGF), which stimulate angiogenesis and granulation tissue formation. Lymphocytes subsequently migrate to the wound site, contributing to extracellular matrix production and collagen remodeling. Disruption of lymphocyte function has been shown to impair wound strength and collagen deposition, highlighting their critical role (Singh et al., 2017; Young & McNaught, 2011).

Inflammation persists until bacteria and debris are cleared. Prolonged inflammation, however, can damage tissue and delay healing. Factors such as lipoxins and arachidonic acid metabolites eventually downregulate the immune response, transitioning the wound to the next phase (Singh et al., 2017; Young & McNaught, 2011).

2.3 Proliferation

The proliferative phase focuses on tissue repair through angiogenesis, granulation tissue formation, collagen deposition, epithelialization, and wound contraction.

2.3.1 Angiogenesis

Angiogenesis is triggered immediately after the formation of the hemostatic plug by growth factors such as PDGF, TGF-β, and fibroblast growth factor (FGF). Hypoxia-induced vascular endothelial growth factor (VEGF) promotes endothelial cell migration and neovascularization, forming a vascular network throughout the wound to restore perfusion (Singh et al., 2017; Young & McNaught, 2011).

2.3.2 Fibroblast Migration

Growth factors from the hemostatic clot stimulate fibroblasts to proliferate and migrate to the wound, a process driven by TGF-β and PDGF. By the third day post-injury, fibroblasts dominate the wound site, producing extracellular matrix proteins (e.g., hyaluronan, fibronectin, and proteoglycans) and synthesizing type III collagen, a hallmark of granulation tissue.

As the matrix matures, fibroblasts transition to myofibroblasts, which contract the wound by connecting to fibronectin and collagen. Peak collagen deposition occurs by day five, forming a palpable "wound ridge." Excessive collagen deposition, however, may result in hypertrophic scarring, particularly in wounds subjected to tension or infection (Singh et al., 2017; Young & McNaught, 2011).

2.3.3 Epithelialization

Epithelialization begins soon after injury as epithelial cells migrate from the wound margins to form a continuous sheet over the wound. This process is mediated by epithelial-mesenchymal transition (EMT), enhancing cellular motility. Cytokines subsequently induce epithelial cells to proliferate and repopulate the wound. In cases of large wounds, secondary intention healing or skin grafting may be required (Singh et al., 2017; Young & McNaught, 2011).

2.3.4 Wound Retraction

Approximately seven days post-injury, wound contraction begins, driven by myofibroblasts. These cells utilize actin and myosin interactions to draw tissue margins together, reducing the wound size at a rate of 0.75 mm per day. Factors such as wound geometry influence contraction speed, with linear wounds contracting faster than circular ones. Dysregulation of this process can lead to deformities or contractures (Singh et al., 2017; Young & McNaught, 2011).

2.4 Remodeling

The final phase of wound healing, remodeling, spans up to two years and results in scar tissue formation. This stage involves the balance of collagen synthesis and degradation, with type III collagen gradually replaced by type I collagen to achieve tissue strength and integrity.

Although the wound never regains full strength, long-term tensile strength reaches approximately 80% of pre-injury levels. Over time, scar vascularity diminishes, leading to a color transition from red to pink to gray (Singh et al., 2017; Young & McNaught, 2011).

Gelatin-based materials have gained attention for their role in wound healing due to their non-toxic, biodegradable, and cost-effective properties. Gelatin-based dressings facilitate hemostasis, support granulation tissue formation, and can be combined with bioactive agents to enhance healing outcomes (Seon Choi et al., 1999; Ulubayram et al., 2002).

This detailed understanding of wound healing provides a foundation for developing innovative therapies and materials that enhance recovery, reduce complications, and improve patient outcomes.

3. Gelatin as a sponge material

Gelatin is a hydrolysis product of the fibrous and insoluble protein collagen. It is widely distributed in nature and forms a major structural component of skin, bones, and connective tissues (Boanini et al., 2010; Kabiri et al., 2011; Seon Choi et al., 1999b; Wang et al., 2020). It is composed of a unique amino acid sequence predominantly featuring glycine, proline, and hydroxyproline, which impart excellent biocompatibility, biodegradability, non-toxicity, and non-carcinogenicity. These attributes, coupled with its affordability, have made gelatin a commercially viable material for use in wound dressings and other biomedical applications (Kabiri et al., 2011; Wang et al., 2020; Yang et al., 2018; Zeugolis et al., 2008). Structurally, gelatin is characterized by its triple-helical molecules

that effectively immobilize water, consisting of repeating glycine-X-Y triplets, where X and Y are typically proline and hydroxyproline (Kabiri et al., 2011; Tomihata et al., 1993).

Gelatin finds widespread use in various biomedical applications due to its non-antigenic nature, distinguishing it from collagen, which exhibits antigenicity under physiological conditions. Additionally, gelatin is more practical than collagen because extracting native collagen is labor-intensive and expensive. This makes gelatin a preferred alternative for developing biomedical products (Seon Choi et al., 1999b; Ulubayram et al., 2001). However, gelatin is rarely used in its native state because of its sensitivity to temperature changes, as it dissolves readily in water at temperatures above 40°C (Khadidja et al., 2017; Kirdponpattara et al., 2017).

In tissue engineering, gelatin is often prepared as a porous, three-dimensional (3D) scaffold. This spongy structure provides ample surface area for cell adhesion, proliferation, and differentiation, facilitating tissue regeneration (Yang et al., 2018). However, because of its water solubility and limited mechanical strength, gelatin requires cross-linking to enhance its stability, mechanical properties, and resistance to hydrolysis (Kabiri et al., 2011; Yeh et al., 2012). Cross-linking is achieved through physical, chemical, or enzymatic methods. Physical techniques such as dehydrothermal treatment, plasma treatment, and ultraviolet irradiation offer limited cross-linking because the reactions occur primarily on the material's surface (Ratanavaraporn et al., 2010). Chemical cross-linking, employing agents like formaldehyde, glutaraldehyde, carbodiimides, genipin, and fructose, provides a higher degree of cross-linking and improved scaffold durability. However, the toxicity of these agents must be carefully evaluated to ensure the biocompatibility of the final product (Kabiri et al., 2011; Yeh et al., 2012). Enzymatic methods, using transglutaminase, tyrosinases, and horseradish peroxidases, offer a safer alternative to chemical agents but may require optimization to achieve comparable effectiveness (Seon Choi et al., 1999b; Ratanavaraporn et al., 2010).

Gelatin-based scaffolds play a pivotal role in tissue engineering by acting as templates that support cell adhesion, extension, proliferation, and differentiation. For optimal performance, scaffolds should exhibit biomimetic properties, excellent biocompatibility, appropriate mechanical strength, and controllable biodegradability to match the physiological needs of the regenerating tissue (Choi et al., 2013; Ko et al., 2012). The pore size and structure of scaffolds are critical factors influencing tissue development, as they regulate cell infiltration and nutrient transport. Advances in scaffold fabrication technologies have enabled precise control over pore structure and size, thus improving tissue regeneration outcomes (Ko et al., 2012; Tai et al., 2007).

Numerous techniques are employed to produce porous 3D biodegradable scaffolds, including gas-forming foam, thermal-induced phase separation, three-dimensional printing, electrospinning, lyophilization (freeze-drying), solvent casting, melt molding, membrane lamination, and particulate leaching (Choi et al., 2013; Ko et al., 2012; Lee et al., 2005). Each technique offers unique advantages, enabling the customization of scaffold properties to suit specific biomedical applications. Additionally, gelatin's hemostatic properties further extend its applications in wound healing, where it accelerates clot formation and aids in acute wound management (Chvapil, n.d.; Seon Choi et al., 1999b).

Gelatin has emerged as a versatile biomaterial for regenerative medicine and tissue engineering due to its excellent properties, affordability, and ease of modification through cross-linking. Advances in scaffold fabrication and cross-linking methods have significantly enhanced the utility of gelatin in promoting tissue regeneration. However, careful evaluation of cross-linking agents' toxicity and optimization of scaffold properties remain essential to ensure the safety and efficacy of gelatin-based biomaterials in clinical applications.

4. Techniques for preparation of gelatin sponge

In the development of tissue engineering scaffolds, many conventional techniques are being followed which include freeze-drying techniques (lyophilization), electrospinning techniques, salt-leaching methods, rapid prototyping, etc. The above methods have been discussed below:

4.1 Freeze-drying techniques or Lyophilization: The freeze-drying techniques also known as lyophilization introduce a method for the production of highly porous 3D scaffolds based on natural polymer, synthetic polymer, and ceramics. Lyophilization is mostly preferred amongst the rest of the techniques in the preparation of polysaccharide scaffolds because it is beneficial for polysaccharides in aqueous media, by dissolving the polymer in the aqueous media and freezing of solution results in the formation of ice-crystal which acts as a template for porogenesis whereas synthetic polymer scaffolds are prepared using organic solvents (Choi et al., 2013; Lee et al., 2005). Properties like pore size, morphology, and structure are manageable using a design mold and can be controlled by some changeable freeze-drying factors including instrumental and solution parameters. It showed an enormous prospect in different tissue engineering. Along with the reduction of freezing temperature, the pore size of the scaffold gets reduced. This technique includes physical processes like dissolution, freezing, and sublimation. Lyophilization removes moisture from the frozen product through a vacuum system and the removal of ice crystals produces that pore in the final product. To improve the properties of the scaffold, the freeze-drying technique can be combined with other methods like salt leaching, gas foaming, gel casting, etc. The most advantageous part of this technique is that it uses water as a solvent material, so no toxic organic solvent can be used as well with

no heat involved, hence, protein, drug, and growth factors can easily be employed (Fereshteh, 2018).

***4.2 Electrospinning technique*:** Electrospinning is a simple and cost-effective method for the formation of high-performance and high-functional nanofibers based on polymers and produces scaffolds with an interconnected pore structure. An electric field with a high voltage range of 10-50 kV is applied to generate an electrically charged jet of polymer solution to facilitate fiber formation. Under the high voltage, a cone shaped-polymer solution droplet is formed that is slowly stretched and on increasing the voltage, a jet is formed from the deformed droplet that moves towards the counter electrode at the end of the process (Agarwal et al., 2008; Mahabub Hasan et al., 2014). The process is very complicated though it seems to be a very simple and easily controllable techniques for the production of nanofibers. In both micro and nanofabrication, the advancement of this technique has powered tissue engineering (Mahabub Hasan et al., 2014).

***4.3 Solvent casting/particulate leaching method*:** Another well-known method for the preparation of highly porous scaffolds like wafers, sponges, etc. with controlled pores, is solvent casting (Chowrasia et al., 2024; Jana et al., 2024) . The particulate leaching technique uses ionic salts, which are generally used as a particulate medium to develop sponges (Ko et al., 2012; Mikes et al., n.d.). Both in solvent casting/particulate leaching and phase separation methods require the use of polar solvents (Harris et al., 1998). In solvent casting techniques, the particles that are generally the chemicals of salts with specific desired dimensions are added to the suitable organic solvent, before the cross-linking process. Then, the mixture of hydrogels and salts is molded into a final three-dimensional scaffold after salt leaching (Ko et al., 2012; Murphy et al., 2002). In the salt leaching technique, a porous polymeric scaffold is obtained for tissue engineering application, by the process where salt particles are dispersed in the polymer solution in an appropriate mold for the formation of solid polymer and salt particle construct. The salt particles are then evaporated and vacant the area to create pores and hence, form a porous polymeric scaffold which can be used in tissue engineering to stimulate the cell growth (Hou et al., 2003). Other common modified scaffold fabrication methods include solvent casting/salt leaching, molding/salt leaching, and gas foaming agent/salt leaching (Ko et al., 2012; Tai et al., 2007). However, these traditional procedures, on the other hand, necessitate the use of organic solvents or high processing temperatures, which, could cause cytotoxicity or harm to the scaffolds' inherent qualities (Ko et al., 2012; Tai et al., 2007).

5. Use of different chemical cross-linking agents

***5.1 Glutaraldehyde*:** Glutaraldehyde is one of the most often used chemical cross-linkers to use with gelatin (Imani et al., 2013; Ulubayram, Aksu, et al., 2002). Among the various cross-linkers, it is mostly used, as the amine groups in gelatin react rapidly with it, as well as it is comparatively inexpensive and easy to handle concerning other cross-linkers as highly soluble in aqueous solution (Bigi et al., 2001; Jayakrishnan & Jameela, 1996; Ulubayram, Aksu, et al., 2002). Glutaraldehyde is assumed to cross-link via intermolecular or intramolecular covalent bonds (Figure 1). It can take place in two ways:

1. Schiff base formation-Glutaraldehyde has an aldehyde group which involves the reaction between free amino groups of lysine or hydroxylysine to produce imine linkages.
2. Aldol condensation takes place between the two adjacent aldehyde groups of glutaraldehyde.

The aldol condensation product is more stable than the Schiff base linkage. The aldehyde group of glutaraldehyde not only interacts with the amino groups but also reacts with the carboxyl group, amido group, and other groups of proteins (Chvapil, n.d.; Furuike et al., 2016; Imani et al., 2013; Jayakrishnan & Jameela, 1996; Ulubayram, Aksu, et al., 2002; Yang et al., 2018).

Between the uncrosslinked and crosslinked sponges, a slight color change is observed from white to yellow (Imani et al., 2013). The polymerization of glutaraldehyde takes place rapidly under alkaline medium of pH 7 to pH 9. Besides sensitivity to pH, polymerization and chemical reaction of glutaraldehyde in aqueous solution also consider temperature, time, molar ratios of reactants as well as exposure to light (Chvapil, n.d.; Jayakrishnan & Jameela, 1996; Speer et al., n.d.; Ulubayram, Aksu, et al., 2002). The use of glutaraldehyde as a cross-linking agent in the production of gelatin sponge is done by either glutaraldehyde vapour or solution. The use of glutaraldehyde solution is mostly preferred compared to glutaraldehyde vapor because cross-linking reaction by glutaraldehyde vapor is time-consuming and cannot penetrate the interior of the biomaterial, thus, resulting in poor cross-linking degree, whereas, cross-linking with glutaraldehyde solution is much faster and with good cross-linking degree (Yang et al., 2018). For the use of clinical purposes, cross-linked gelatin should be non-toxic. Though, it has been known that gelatin itself is non-toxic, but glutaraldehyde as cross-linkers cause toxicity. According to the literature, the non-reacted aldehydes group in the polymerization reaction may be the cause of reporting glutaraldehyde cross-linked biomaterials as toxic. It was also reported that glutaraldehyde concentrations higher than 0.05% (v/v) as cross-linking agents are highly cytotoxic (Imani et al., 2013; Ulubayram, Aksu, et al., 2002). Among other aldehydes used as crosslinkers, glutaraldehyde has many merits despite its toxicity and hence, it has been clinically acceptable (Imani et al., 2013; Jayakrishnan & Jameela, 1996; Ulubayram, Aksu, et al., 2002).

5.1.1EDC: Carbodiimides are allene structured-unsaturated compounds and are used to cross-link gelatin; among all, water-soluble 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC) is

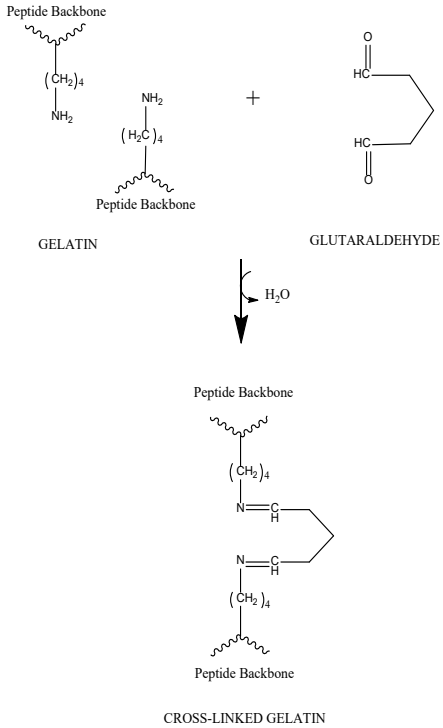

Figure 1. Schematic presentation of gelatin cross-linking with glutaraldehyde

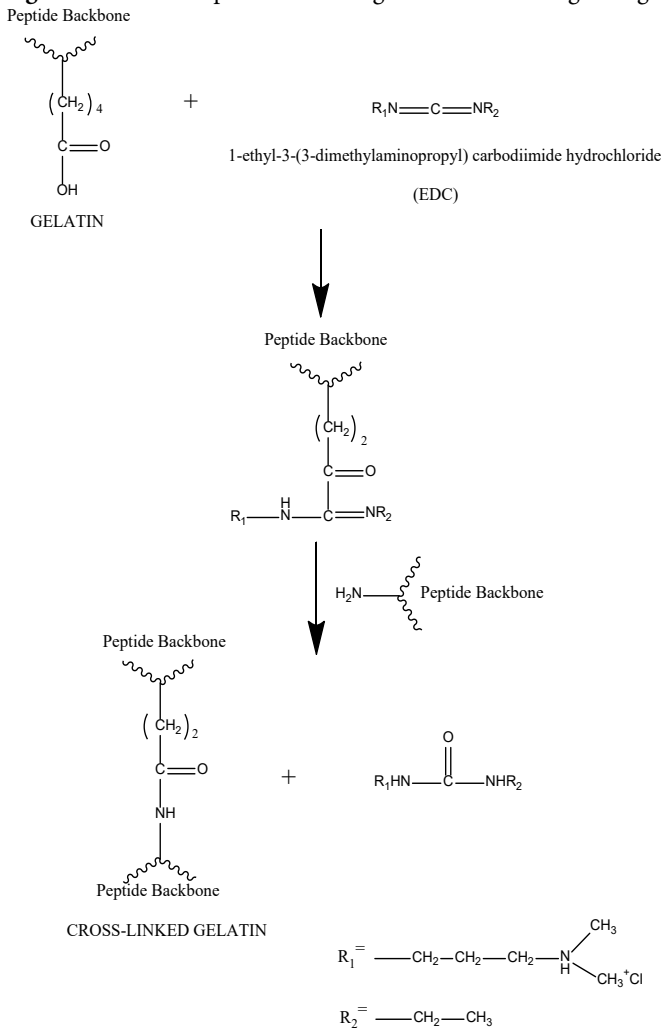

Figure 2. Schematic presentation of gelatin cross-linkage with EDC

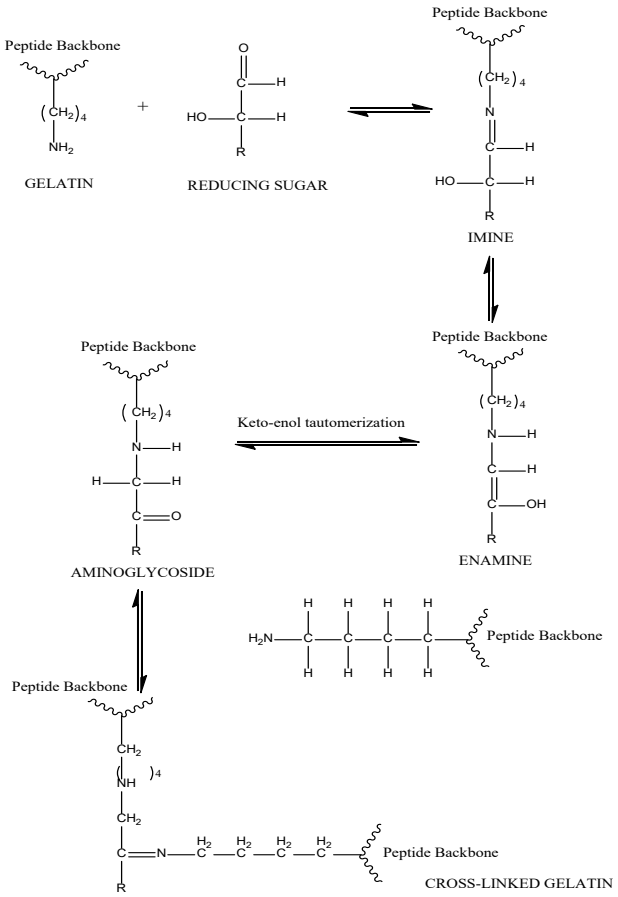

Figure 3. Schematic presentation of gelatin cross-linking with reducing sugars

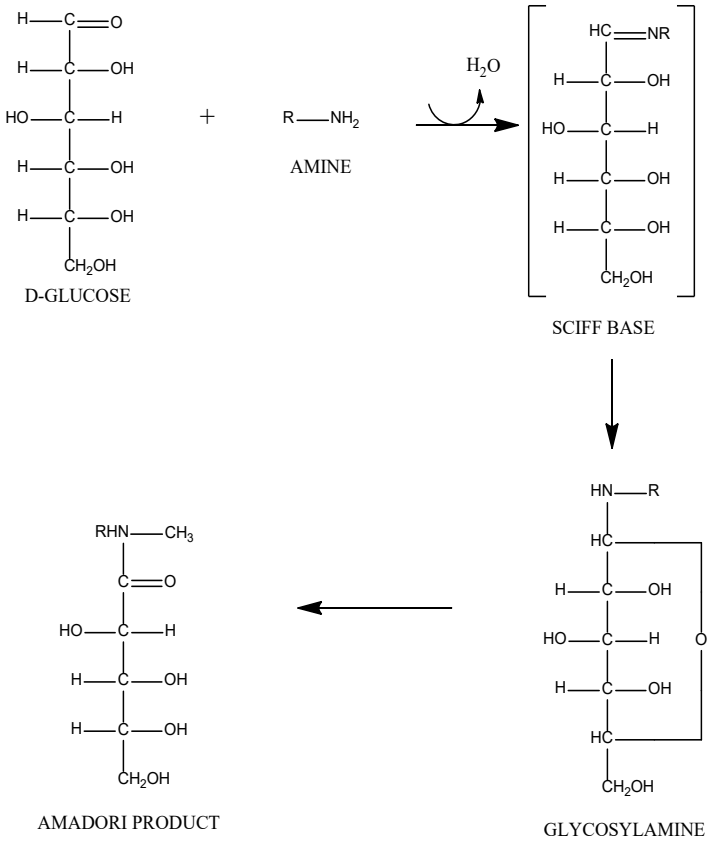

Figure 4. Amadori rearrangement: Maillard reaction with D-Glucose

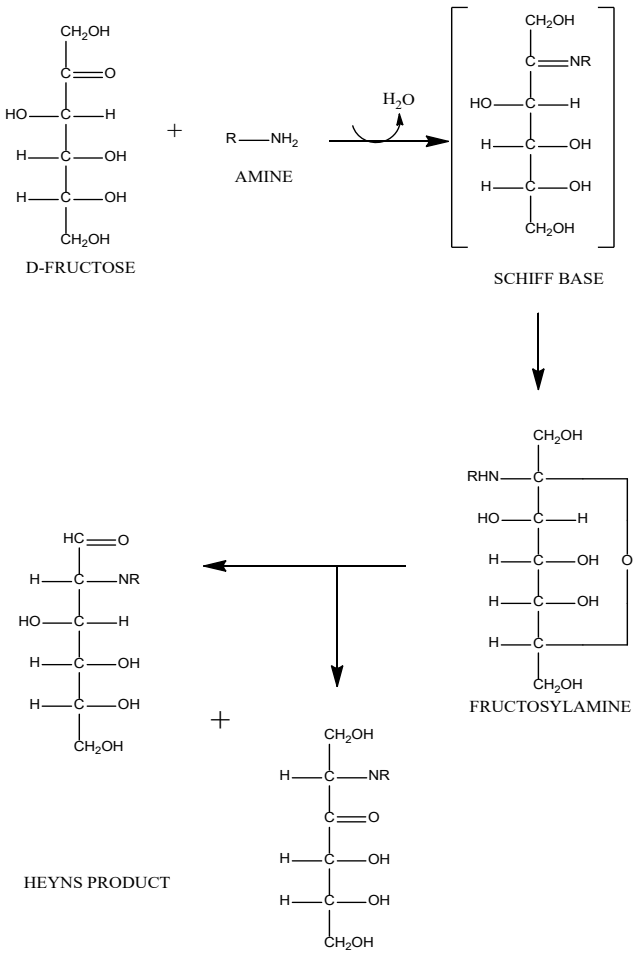

Figure 5. Heyns rearrangement: Maillard reaction with D-Fructose

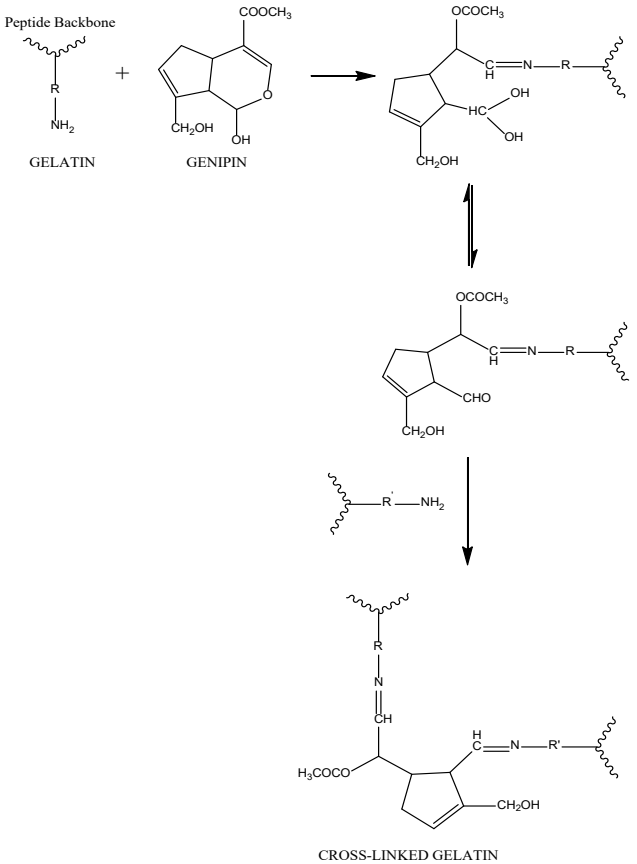

Figure 6. Schematic presentation of gelatin cross-linkage with genipin

preferably used. EDC is widely used in various amino-acid-based biomaterials. In accordance with the toxicity of cross-linking agent, it is non-toxic and biocompatible because it is not incorporated into the cross-linked gelatin sponge structure, but simply changed into a water-soluble derivative of urea (Nakajima & Ikada, 1995; Seon Choi et al., 1999b, 1999a; Tomihata et al., 1993; Yang et al., 2018). Thus, it may be considered that the gelatin sponge with EDC as a cross-linking agent shows the highest biosafety as the unreacted EDC and the substituted urea molecule can be completely removed from the crosslinked biomaterials. Furthermore, when compared to EDC, the soluble urea derivative's cytotoxicity was determined to be relatively low. (Seon Choi et al., 1999b, 1999a; Tomihata et al., 1993, 1996; Ulubayram, Aksu, et al., 2002). It is reported that water-soluble carbodiimides are more effective than water-insoluble carbodiimides and mostly available water-soluble molecular form of EDC is 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Tomihata et al., 1993, 1996; Ulubayram, Aksu, et al., 2002). In the cross-linking of gelatin with EDC (Figure 2), EDC activates the free carboxylic acid group of glutamic or aspartic acid form amide linkage with the amino groups of lysine of the same or another polypeptide chain and releases a urea product that is soluble and avoids incorporation of any foreign bonds into the cross-linked matrix. (Powell & Boyce, 2006; Seon Choi et al., 1999a; Ulubayram, Aksu, et al., 2002; Yang et al., 2018; Yeh et al., 2012).

To increase the mechanical strength of gelatin sponge approximately 0.025-0.05w/w is used and the optimal temperature for the reaction ranges between 15-25°C. However, it is well known that EDC is an expensive reagent and easily loses its activity in aqueous solution, leading to low mechanical strength, though it shows greater efficacy than the un-crosslinked gelatin sponge (Kabiri et al., 2011; Nakajima & Ikada, 1995; Seon Choi et al., 1999b, 1999a; Tomihata et al., 1996; Yang et al., 2018). It was also reported that the cross-linking of gelatin with EDC shows similar effectivity as that of glutaraldehyde as the cross-linking agent, which is used widely despite its toxicity (Tomihata et al., 1996).

5.2 Reducing Sugar: The addition of reducing sugars increases the yield of cross-linked products, thus, it may be used as cross-linking agents, and, additionally, they are non-toxic and inexpensive compared to other cross-linking agents like glutaraldehyde and EDC. Glucose and fructose are mostly reported to be used as cross-linking agents which follows the Maillard reaction for cross-linking gelatin. In the Maillard reaction, the carbonyl group of the reducing sugar undergoes a condensation reaction with the free amino group present in the amino acid of gelatin forming an aminoglycoside bond which further reacts with another amino group that leads to cross-linking the network structure (Figure 3) (Cortesi et al., 1998; Kirdponpattara et al., 2017; Masutani et al., 2014; Ulubayram, Aksu, et al., 2002).

5.2.1 Glucose: For the biocompatibility of the gelatin sponge material, glucose is an effective cross-linking reagent. It is non-toxic and it can also react and polymerize at high temperatures. It was reported that 15w/w glucose solution are preferably used for highest degree of swelling and porosity, a level higher than 15w/w makes the biomaterial stiff and brittle (Kirdponpattara et al., 2017; Siimon et al., 2014). The formation of covalent interaction between the glucose and the amine group of gelatin is important for a thermostable compound, Maillard reaction can form that covalent bond (Figure 4). The reaction combined with thermal treatment or freeze-drying techniques improves the cross-linking degree of the gelatin sponge (Kirdponpattara et al., 2017; Masutani et al., 2014; Siimon et al., 2014).

5.2.2Fructose: Reducing sugar fructose has also been considered biocompatible eliminating the risk of toxicity (Cortesi et al., 1998; Ulubayram, Aksu, et al., 2002). In the Maillard reaction, a specific concentration of fructose, results in qualitative and quantitative differences (Protein Fructosylation: Fructose and the Maillard Reaction, 1993). Maillard reaction involves a series of complex reactions, wherein the first stage, condensation of the carbonyl group of reducing sugar with the α-amino acid takes place. The resulting glycosylamine undergoes an Amadori rearrangement, which in fructose is termed Heyns rearrangement (Figure 5). Moreover, it is also reported that the Maillard reaction of protein by fructose is much more rapid than with glucose (Protein Fructosylation: Fructose and the Maillard Reaction, 1993).

***5.2.3Genipin*:** Genipin is an aglycone obtained from an iridoid glucoside, its parent molecule geniposide, that may be extracted from the fruits of *Gardenia jasminoides* Ellis by modern microbiological processes (Bigi et al., 2002; de Clercq et al., 2016; Gomes et al., 2013; Liang et al., 2004; Muzzarelli, 2009). It is widely used in herbal medicine as an antiphlogistic and cholagogue. Genipin is a naturally occurring cross-linking agents and it is favourable due to its promising characteristics, which includes low toxicity, higher biocompatibility, and its ability of self-polymerization. It was reported that genipin shows 10,000 times less toxicity compared with aldehyde and epoxy crosslinkers. It also increases the stability of cross-linked gelatin by both thermally and structurally (de Clercq et al., 2016; Mekhail et al., 2014; Nickerson et al., 2006; Panzavolta et al., 2011). Genipin covalently binds with the amino acids present in the gelatin, generally, lysine and in a lower degree with hydroxylysine and arginine, by SN^2 nucleophilic substitution reaction (Figure 6). The formation of cross-linked gelatin occurs in two stages, in the first stage, it is initiated with the nucleophilic attack by the amino group of the gelatin on the 3rd carbon of the genipin, which results in an opening of the genipin ring and forms an aldehyde group. Then, this resulting aldehyde group, attacked by the attached secondary amino group. Dimerization occurs in the second stage through radical reaction.

Thus, genipin can form intramolecular crosslinks within a gelatin molecule or intermolecular cross-links with the adjacent gelatin molecules and gives a cross-linked product having a heterocyclic structure (Chang et al., 2003; de Clercq et al., 2016; Liang et al., 2004; Nickerson et al., 2006). It is reported that, because of the bulky heterocyclic structure, crosslinking with ginipin is more difficult in relaxation compared with the glutaraldehyde-crosslinking, and thus the swelling ratio is less prominent, and water-vapor transmission rate is slower (Chang et al., 2003).

***5.2.4Other chemical cross-linking agents*:** Other compounds that can be cross-linked with gelatin include hexamethylene diisocyanate (HMDC), 2-chloro-1-methylpyridinium iodide (CMPI), and two acyl azide compounds hydrazine and diphenyl phosphorylazide (DPPA) (Rault et al., 1996; Yeh et al., 2012).

Hexamethylene diisocyanate (HMDC) is a bifunctional molecule. Cross-linkage by HMDC is due to the primary reaction between amino groups present in the protein similar to glutaraldehyde. It is water-insoluble and highly reactive in aqueous solution; hence, surfactant is used with the cross-linking reaction (Naimark et al., 1995). It enhances the thermal stability of collagen sponge (Rault et al., 1996). As the reaction is similar to that of glutaraldehyde, it can be used as an alternative, but further studies need to be carried out.

CMPI is a useful chemical reagent for the activation of carboxyl groups. It has some promising characteristics that includes high yield of the cross-linked product, water-soluble, shows low toxicity, need simple reaction conditions and comparatively less expensive. Cross-linking formation is either by forming intramolecular linkage within the same gelatin molecule or intermolecular linkage between the adjacent gelatin molecules. The carboxyl group of aspartic acid or glutamic acid forms an amide bond with the amino group of lysine residues (Rault et al., 1996).

Cross-linking with acyl azides reported high thermal stability. Compared with HMDC, hydrazine shows an increase in thermal stability. Among the two acyl azides, DPPA has high thermal stability (Rault et al., 1996).

6. Applications

Gelatin is commercially available for use in wound dressing materials, but it lacks sufficient antibacterial activity on its own. To address this limitation, incorporating antibacterial agents into gelatin sponges has been shown to effectively eliminate bacteria and promote wound healing (Wang et al., 2020). Marketed formulations such as SURGISPON and HEMOSPONGE are notable examples of this approach. Ideal wound dressings must possess several key properties, including biocompatibility, non-toxicity, and the ability to swell and absorb water effectively. Due to these characteristics, gelatin sponges are well-suited for applications in skin tissue engineering, wound dressing, and surgical procedures (Kabiri et al., 2011; Kirdponpattara et al., 2017; Wang et al., 2020).

In surgical contexts, the ability to facilitate blood clot formation and achieve hemostasis is critical. Rapid clotting not only prevents infections but also initiates tissue repair and healing processes. Gelatin is particularly valuable in this regard due to its high hemostatic effect, making it advantageous for surgical applications (Kabiri et al., 2011). For example, in transsphenoidal surgeries, a gelatin sponge combined with fibrin glue has been successfully used to prevent cerebrospinal fluid (CSF) leaks. In this procedure, a fibrinogen solution is applied to a gelatin sponge, followed by a diluted thrombin solution, creating a fibrin-soaked sponge for effective sealing (Nagahama et al., 2019).

The versatility of gelatin sponges extends to postoperative recovery applications. A case study reported the use of a gelatin sponge loaded with a mixture of three drugs for intraoperative nerve root blocks, which significantly promoted early postoperative recovery in patients with lumbar disc herniation (LDH) (Du et al., 2018; Jevotovsky et al., 2018). Such innovative uses demonstrate the adaptability of gelatin-based materials in various medical contexts, enhancing their appeal as a multifunctional biomaterial.

Gelatin sponges, particularly when enhanced with antibacterial agents or combined with other therapeutic materials, hold significant potential for wound care, surgical applications, and tissue engineering. Their biocompatibility, hemostatic properties, and capacity for functional modifications underscore their utility in advancing medical treatments.

7. Conclusion

Gelatin cross-linking is essential in biomedical applications to enhance mechanical strength, improve hydrolysis resistance, and increase stability. During the production of gelatin sponges, selecting an appropriate cross-linking agent is critical. The agent must be non-toxic and cost-effective to ensure its suitability for commercial use in wound dressings and other biomedical applications.

Among commonly used cross-linkers, glutaraldehyde is a popular aldehyde due to its effectiveness. However, it is highly cytotoxic, limiting its practicality for applications requiring biocompatibility. In contrast, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), a water-soluble carbodiimide, achieves a good degree of cross-linking with gelatin while being less cytotoxic than glutaraldehyde. Another promising alternative is genipin, a naturally derived cross-linking agent, which is approximately 10,000 times less toxic than glutaraldehyde. Despite their advantages, both EDC and genipin are relatively expensive compared to other cross-linking agents.

Reducing sugars, such as glucose and fructose, offer a promising solution as cross-linking agents. These sugars are non-toxic,

biocompatible, and inexpensive, making them attractive candidates for the cross-linking of gelatin in biomedical applications. Their affordability and safety make them particularly suitable for large-scale production of gelatin-based materials for wound care and tissue engineering.

Author contributions

I.S. contributed to the conceptualization of the study and the preparation of the manuscript draft. B.K.J. was involved in the methodology design and data analysis. N.R.G. assisted in data collection and validation. H.P. contributed to the interpretation of the results and manuscript editing. M.S. provided technical support and reviewed the manuscript critically. B.M. supervised the project, ensured the integrity of the research process, and approved the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgment

The authors were grateful to their department.

Competing financial interests

The authors have no conflict of interest.

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