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To Cross-Link or Not to Cross-Link? Cross-Linking Associated Foreign Body Response of Collagen-Based Devices

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Collagen-based devices, in various physical conformations, are extensively used for tissue engineering and regenerative medicine applications. Given that the natural cross-linking pathway of collagen does not occur *in vitro*, chemical, physical, and biological cross-linking methods have been assessed over the years to control mechanical stability, degradation rate, and immunogenicity of the device upon implantation. Although *in vitro* data demonstrate that mechanical properties and degradation rate can be accurately controlled as a function of the cross-linking method utilized, preclinical and clinical data indicate that cross-linking methods employed may have adverse effects on host response, especially when potent cross-linking methods are employed. Experimental data suggest that more suitable cross-linking methods should be developed to achieve a balance between stability and functional remodeling.

Introduction

Collagen is one of the major structural extracellular matrix (ECM) proteins in mammals, constituting 20% to 30% of the total body proteins. To date, 29 different collagen genes have been identified,[1] with all types sharing a unique and common triple helical configuration with a repeated $[Gly\text{-}X\text{-}Y]_n$ sequence, where X is often proline and Y is frequently hydroxyproline.[2,3] Among the different collagen types, collagen type I, predominantly localized in the skin, tendon, cornea, and bone, is the most abundant in the body and consequently the most widely studied. Collagen-based materials, in the form of tissue grafts and reconstituted scaffolds, are attractive for biomedical applications, as due to their natural composition and their well-tolerated degradation products are perceived by the host as normal constituents rather than as foreign matter and therefore provide an acceptable host response.[4] In addition to their superior mechanical properties, collagen-based devices provide instructive cues to the cells, promoting this way functional tissue repair and regeneration.[5,6] It is therefore not surprising that the collagen-based medical device market is estimated to reach 3.7 billion US dollars by 2017.[7]

The natural cross-linking pathway of lysyl oxidase is responsible for mechanical resilience of tissues and their proteolytic resistance.[8] The harsh extraction/purification methods,[9] scaffold fabrication technologies,[10] and the subsequent sterilization methods[11] necessitate the introduction of exogenous cross-links (chemical, physical, or biological in nature) into the molecular structure of collagen implants to control their degradation rate and enhance their mechanical stability.[12–14] However, such cross-linking approaches are associated with numerous shortfalls as a function of the cross-linking density/method, including cytotoxicity,[15,16] calcification,[17–19] and foreign body response.[20,21]

Herein, we discuss the host/macrophage response, as a function and extent of the cross-linking density/method employed to stabilize collagen-based devices. We recognize that the extent of cross-linking can be assessed by denaturation temperature, quantification of free amine groups, swelling, mechanical properties, and/or resistance to enzymatic degradation. However, denaturation temperature is customarily used to assess cross-linking density due to the simplicity and accuracy of the technique. Thus, herein we define collagen materials that are slightly cross-linked, moderately cross-linked, and heavily cross-linked as those that have exhibited denaturation temperature of $< 65°C$, 65–70°C, and $> 70°C$, respectively.

Collagen Cross-Linking Methods

The fundamental principle of collagen cross-linking is the formation of covalent bonds between collagen molecules using chemical or natural moieties, which bind either to the

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free amine or carboxyl groups of collagen. The most commonly used chemical cross-linking reagents are aldehydes (e.g., glutaraldehyde [GTA]),[22] isocyanates (e.g., hexamethylene diisocyanate [HMDI]),[23] and carbodiimides (e.g., 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide [EDC]).[24] Photoreactive agents (e.g., rose Bengal,[25] riboflavin[26]), carbohydrates (e.g., ribose,[27] glucose[28]), and plant extracts (e.g., genipin,[29,30] oleuropein,[31] and *Myrica rubra*[32]) have also been used, but to a lesser extent. Recently, the influence of polyethylene glycol (PEG) polymers that can vary in molecular weight, degree of branching, and terminal groups are under intense investigation as means to cross-link and functionalize collagen-based devices.[33–36] To avoid cytotoxic effects associated with the chemical cross-linker itself of its residue, physical (e.g., dehydrothermal [DHT][37–42] and UV irradiation[41,43–46]), and biological (e.g., transglutaminase[47–51]) methods have also been assessed.

Each cross-linking method has demonstrated a different degree of structural and mechanical stability, largely attributed to the different cross-linking mechanisms, concentration, and exposure time. Cross-linking with GTA involves an heterogeneous cross-linking distribution that occurs only on the surface of the fibrils and fibers, which leads predominantly to intermolecular cross-links connecting distant collagen molecules.[52] The ability of GTA to self-polymerize probably accounts for its effectiveness as a cross-linker,[53] while the effectiveness of formaldehyde as a cross-linker depends on its abundance rather than on the individual lengths of the cross-links.[54] The bifunctional cross-linker HMDI has been used as an alternative to GTA, due to its superior cytocompatibility and proportional mechanical stability.[55,56] Carboxyl group cross-linking, through carbodiimide[24,57–60] or acyl azide[61–69] methods, has been extensively utilized as an alternative to GTA/HMDI; such approaches are more cytocompatible as they do not introduce foreign cross-linking molecules; less resistant to proteolytic attack; and less susceptible to calcification; however, the produced devices are not as strong as such methods can couple proximate collagen molecules.[70,71] To increase efficiency and avoid denaturation, the DHT treatment requires high vacuum to reduce the water content before heating at over 100°C for several hours and is usually followed by an EDC step.[41,72] Mimicking the *in vivo* collagen cross-linking, tissue-type (Ca^{2+} dependent) or microbial (Ca^{2+} independent) transglutaminase (biological method) has been used to covalently cross-link ECM proteins resulting in a covalent γ-glutamyl-ε-lysine isopeptide bond.[48] Collagen-based materials that have been cross-linked with mammalian or microbial in origin transglutaminases have demonstrated a moderate increase in denaturation temperature, mechanical resilience, and biological stability, compared to chemical cross-linking approaches.[73–76]

Physiological Wound Healing

The wound healing process is the innate response of all tissues to any injury or device implantation. It is a complex process that is regulated by several cell types, growth factors, and cytokines that direct the four overlapping phases (Figs. 1 and 2), namely hemostasis, inflammation, new tissue formation, and tissue remodeling.[77]

Hemostasis occurs immediately after injury or device implantation. Released factors from the tissue induce platelets to secrete clotting factors (e.g., mainly serotonin, thromboxane, platelet-derived growth factor [PDGF], transforming growth factor beta [TGF-β]) to promote coagulation and to develop a fibrin clot.[78] This provisional fibrin matrix acts as a scaffold for further cell migration. Simultaneously to the formation of the fibrin clot, a dynamic interaction between blood plasma proteins and the device surface occurs and a provisional matrix around the biomaterial surface is developed; this event is known as the Vroman effect.[79] The initial protein adsorption depends on the surface properties of the device, including wettability,[80,81] surface charge/chemistry,[82,83] topography/roughness,[84–86] and stiffness,[87] which modulate the cell/inflammatory response and subsequent wound healing.

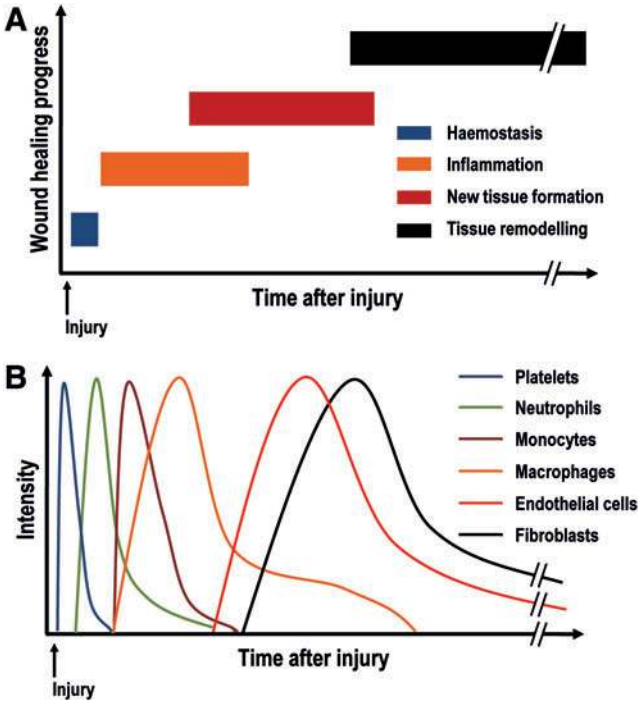

FIG. 1. Temporal distribution of the four overlapping wound healing phases **(A)** and associated cell type **(B)**. Color images available online at www.liebertpub.com/teb

Following hemostasis, acute inflammation begins between 24 and 48 h after injury. This phase is characterized by the recruitment of neutrophils and mast cells in response to chemokines (cytokines with chemoattractive properties) and other chemoattractants (mainly interleukin [IL]-1, IL-6, IL-8, monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP]-1, and tumor necrosis factor alpha [TNF-α]).[88,89] Neutrophils and mast cells phagocytize foreign material, bacteria, dead cells, and damaged matrix within the wound. The presence of contaminants/foreign matter at the wound bed increases the neutrophil presence, reactive oxygen species (ROS), and cytokine signaling.[88,90] Neutrophils secrete TGF-β, PDGF, platelet factor 4 (PF4), and IL-1 to recruit further mast cells and monocytes. Mast cells secrete histamine and other cytokines that recruit leukocytes into the injury site. After 48–72 h, monocytes migrate and differentiate into macrophages, which secrete TNF-α, IL-6, RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1, and MIP-1, to recruit further macrophages and dominate the cell population at the injury site.[91] Initially,

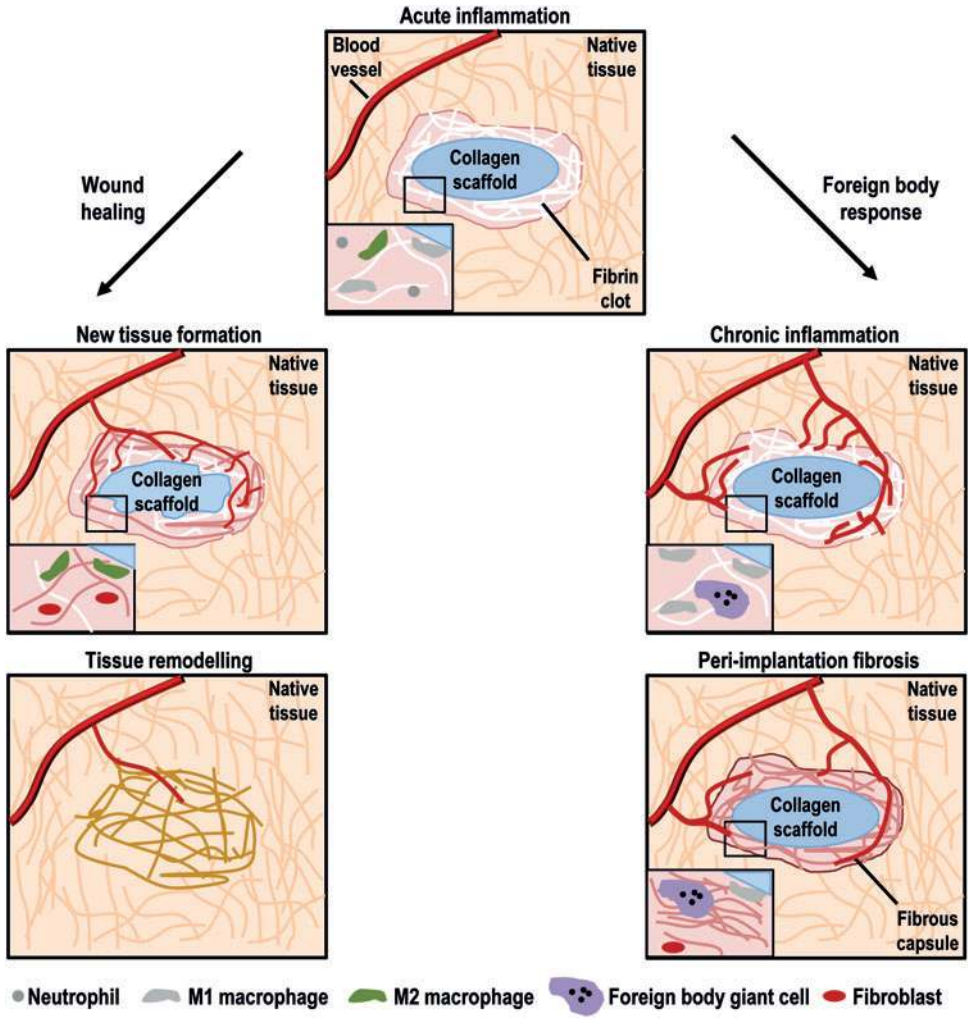

FIG. 2. Acute inflammation is characterized by the presence of neutrophils, monocytes, and macrophages. Depending on the resolution of the acute inflammation, injury repair could lead to a wound healing process or a classical foreign body response. In wound healing, M2 macrophages attract fibroblast and endothelial cells to secrete a new vascularized tissue. This connective tissue replaces fibrin clot and degraded scaffold. Tissue remodeling is the last healing and could continue for over a year. In foreign body response, acute inflammation persists over time and M1 macrophages aggregate into foreign body giant cells (FBGCs). M1 macrophages and FBGCs fail to degrade the foreign scaffold, resulting in fibroblast recruitment and deposition of a fibrous connective tissue around the scaffold (peri-implantation fibrosis). Color images available online at www.liebertpub.com/teb

macrophages are mainly M1 phenotype, a proinflammatory or classically activated phenotype. M1 macrophages attack potential pathogens or phagocytize at the wound site, as a response to interferon gamma (IFN-γ), TNF-α, or bacterial lipopolysaccharides. Gradually, M1 macrophages change to M2 phenotype, an anti-inflammatory or alternatively activated phenotype. M2 macrophages have been described as displaying different subphenotypes or roles, which are anti-inflammatory (M2a), immunoregulatory or homeostatic (M2b), and prowound healing (M2c).[92–94] The macrophage phenotype switch is induced by cytokines, such as IL-4, IL-10, and IL-13, and functional reconstruction depends on the timing of this change. Macrophage activation and polarization is crucial in the coordination of the later inflammation and regeneration phases.[95,96] Thus, macrophage polarization and activation is at the forefront of scientific investigation, with various studies aiming to modulate it using biophysical cues (e.g., architectural features,[97] topographical patterns[85]), biochemical signals (e.g., incorporation of glycosaminoglycans[98] or drugs[99]), and biological means (e.g., gene therapy with lipoplexes[100] or polyplexes[101]).

New tissue formation begins 2–10 days after injury and is identified by migration and proliferation of different cell types that produce a new ECM and form the initial wound. In skin, for example, keratinocytes migrate over the dermis and restore the barrier function of the epidermis,[88] while fibroblasts, attracted by macrophage cytokines, migrate to the injury site from the wound edge or bone marrow and differentiate into myofibroblasts, contributing to the wound contraction.[102] During this stage, fibroblasts and myofibroblasts interact, migrate, proliferate, and secrete ECM proteins to replace the fibrin matrix and form new tissue, predominantly constituted by collagen type III and smaller amounts of fibronectin, elastin, and proteoglycans.[88,103] Meanwhile, macrophages and fibroblasts secrete vascular endothelial growth factor (VEGF) and FGF2 that promote endothelial and progenitor cells to produce new blood vessels, respectively.[103,104] These new vessels start out from pre-existing vessels adjacent to the wound.[88]

Tissue remodeling significantly increases 2–3 weeks after injury and could continue for over a year. The remodeling phase is characterized by different cell types undergoing reduction in cell activity and apoptosis and the creation of mature blood vessels. Collagen type III is gradually replaced by collagen type I, an event mainly controlled by matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs), and mechanical stress and strain. The properties of the tissue are partially recovered, but the new tissue hardly ever reaches the preinjury state; for example, the dermis reaches up to 70% of its preinjury tensile strength.[88]

Impaired Wound Healing

Multiple potential factors, local (e.g., injury size, infection, device properties, and degradation products) or systemic (e.g., nutrition, age, health state)[105] in nature, can interfere with one or more wound healing stages resulting in improper or impaired wounds. The most common impaired wounds after device implantation are delayed acute wounds, chronic wounds, and peri-implantation fibrosis, the hallmark of which is a patch of inflammatory cells, mainly macrophages and foreign body giant cells (FBGCs), and a disorganized ECM, mostly collagen.[88,105] In severe burns, immunosuppression is brought about due to suppression of T-cell proliferation, large macrophage activation, and a high amount of proinflammatory cytokine and free radicals that predispose patients to impaired healing, infection, and systemic organ failure.[106,107]

Local factors, such as bacterial contamination and foreign material that cannot be cleaned or degraded, respectively, induce the inflammatory cells (monocytes, M1 macrophages, and FBGCs) to remain at the device's interface, prolonging the inflammation phage to over months or years and leading into chronic inflammation and healing failure.[88] Systemic factors are associated with the overall health/disease state of the patient. Increased age is often associated with impaired wound healing. For example, in healthy older adults, wound healing suffers a temporal delay associated with dysfunction of macrophage phagocytic capacity[108] and polarization,[109] delayed angiogenesis,[110] and delayed collagen synthesis and re-epithelization.[111] Obesity, which nowadays affects over 500 million people worldwide,[112] induces hypoxia and a high infection rate due to skin folds and partial suppression of T-cell function[113,114]; prompts wound dehiscence by increasing tension on wound site[115]; and alters the adipocyte and macrophage ratio in adipose tissue, inducing increased production of adipocytokines (e.g., TNF-α, IL-6, IL-8, and MCP-1).[116] This increase of adipocytokines, in combination with the activation of granulocytes and monocytes that secrete free radicals and proteolytic enzymes,[114] compromises the wound healing process. Diabetes, with over 382 million sufferers worldwide in 2013,[117] increases ROS production and reduces antioxidant secretion, leading to oxidative stress,[118] which when combined with the hypoxic stress of diabetic wounds,[119] leads to an increased inflammatory response.[105] Furthermore, diabetic patients have several dysregulated cellular functions, including reduction of inflammatory cell recruitment,[120] limited bacterial phagocytosis,[121] and dysfunction of macrophage polarization (maintaining a strong M1 marker expression and function[122] or fibroblast dysfunction[123]), which result in an unbalanced expression of growth factors and MMPs that inhibit new tissue formation,[124] compromising physiological wound healing.

Methods for Assessing *In Vitro* Inflammatory Response to Collagen-Based Devices

Although numerous cells (e.g., macrophages,[22] monocytes,[125] neutrophils,[126] leukocytes,[23] and dendritic cells[127]) are employed to study the *in vitro* inflammatory response to biomaterials, macrophages appear to be the preferred cell population for collagen-based devices. This may be due to the determinant role of macrophages in the resolution of inflammation and wound healing and the availability of techniques to characterize macrophage subpopulations and response.[128] To date, the most reliable macrophage sources are those isolated from human peripheral blood mononuclear cells (PBMCs)[98] and immortalized cell lines, such as the human-derived leukemic monocyte cell line (THP-1)[101]; the human leukemic lymphoma monocyte cell line (U937)[22]; and the mouse leukemic monocyte–macrophage cell line (RAW264.7).[129] The advantages of immortalized macrophage cell lines include higher accessibility; lower cell phenotype variability; unnecessary addition of inflammatory mediators in media to prevent apoptosis; and cryopreservation without a detrimental effect on cell viability and differentiation.[130,131] Nonetheless, immortalized cell lines suffer from certain cell dysfunctions, such as adapted growth in culture, reduced cell–cell interaction, and decreased protein secretion.[132]

Our understanding of the host response to collagen-based materials is largely attributed to experimental data on macrophage activation and polarization (Fig. 3). Macrophages express different surface markers according to each subpopulation; M1 macrophages are positive for cluster of differentiation 80 (CD80), CD86, and CCR7, while M2 express CD163 and CD206.[92,95] Furthermore, the different macrophage subpopulations direct inflammation and tissue repair by secreting cytokines and other reactive species. Specifically, M1 macrophages produce proinflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-23, and TNF-α, while M2 macrophages secrete IL-1ra, IL-4, IL-10, IL-13, VEGF, TGF-β, and arginase.[92–95] M1 macrophages regulate inflammation and collagen-based devices degradation by the secretion of nitrites, ROS, and MMPs.[22,133]

In Vitro Assessment of Inflammatory Response to Collagen-Based Devices

Cross-linked collagen-based materials have been shown to preferentially alter macrophage response *in vitro* (Table 1). GTA cross-linked decellularized bovine pericardium (noncommercial material) induced moderate fibroblast cytotoxicity and THP-1 macrophage activation, which secreted a higher amount of TNF-α and IL-6 than the noncross-linked counterpart.[134] Additionally, this same material altered U937 macrophage morphology (cell area reduction and disrupted membrane), reduced attachment and viability, increased release of proinflammatory cytokines (TNF-α and IL-6), and changed MMP pattern secretion (upregulated MMP-1 and downregulated MMP-2 and MMP-9), while EDC cross-linked pericardium reduced the release of proinflammatory cytokines, altered MMP pattern, and induced rounded macrophage morphology,[22] indicative of M1 macrophages.[135] Moreover, PBMCs released a higher amount of TNF-α and IL-6 than IL-10, when cultured on noncommercial GTA cross-linked porcine pulmonary valves.[136] With regard to reconstituted collagen materials, noncommercial EDC cross-linked collagen sponges have been shown to increase *in vitro* resistance to degradation by macrophages; however, these sponges promoted RAW 264.7 macrophage aggregation to form FBGCs that gradually degraded the sponges.[137] When incubated with human primary monocytes/macrophages, commercially available slightly cross-linked (HMDI) porcine dermis grafts (Permacol™ Surgical Implant; Covidien, denaturation temperature

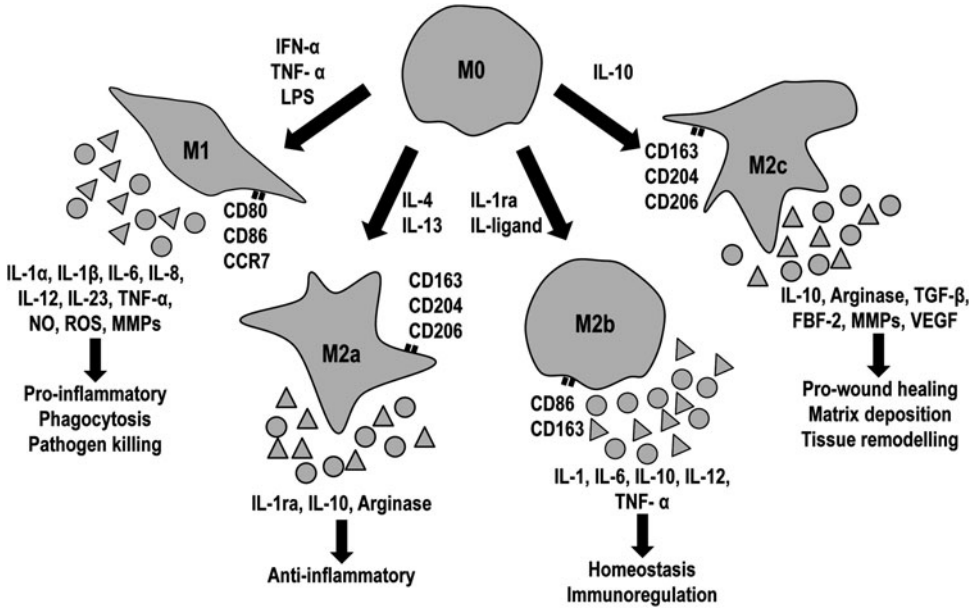

FIG. 3. Macrophages polarized from M0 (non-polarized) to M1 (proinflammatory) or M2 (M2a, anti-inflammatory; M2b, homeostatic; M2c, wound healing) depending on inducing signals. Each macrophage subpopulation expresses different surface markers, cytokines, and reactive species. CD, cluster of differentiation; FBF-2, puf-domain RNA-binding protein; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinases; NO, nitric oxide; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

of 60–61°C,[138,139] while its noncross-linked counterpart has denaturation temperature of 56–57°C[140]) induced low amount of proinflammatory (TNF-α, IL-1β, IL-6, MCP-3, MIP-1α) and anti-inflammatory (IL-1ra, CCL18, MIP-4) cytokines, when compared to other synthetic materials used for soft tissue repair, showing a low M1/M2 protein secretion index.[141] Moreover, Permacol materials did not alter *in vitro* leukocyte viability, activation, and ROS expression. When they were exposed to fresh human peripheral whole blood, they behaved similarly to their noncross-linked counterparts.[23] As EDC treatment, noncommercial DHT cross-linked collagen sponges induced FBGC formation, although the treatment increased the enzymatic resistance.[137]

Macrophage activation has also been associated with release of chemicals/processing by-products and surface modification. Specifically, released by-products from HMDI and EDC cross-linked porcine dermis grafts (Permacol, and CollaMend™ FM Implant; Bard, denaturation temperature at 66°C[138,139]) were associated with the increase of proinflammatory (IL-1β, IL-6, IL-8) and vascular (VEGF) cytokine expression of human PBMCs.[142] However, a recent publication questioned this theory, as no cross-linking agent traces were detected by nuclear magnetic resonance spectroscopy on conditioned media with a noncommercial GTA cross-linked collagen scaffold, and put forward the notion that the increase of proinflammatory cytokine expression

Table 1. *In Vitro* Inflammatory Response Associated with Cross-Linked Collagen-Based Materials

Cross-linking agent	*Summary of* in vitro *results*	*References*
GTA (i.e., Peri-Guard® & noncommercial materials)	Alteration of macrophage morphology (cell area reduction and membrane disruption) Reduction of macrophage attachment and viability Upregulation of proinflammatory cytokines Alteration of MMP secretion	22,134,136
HMDI (i.e., Permacol™ & noncommercial materials)	Increase of enzymatic resistance Moderate upregulation of proinflammatory and angiogenic factors/cytokines No alteration of leukocyte behavior or release of reactive oxygen species	23,141,142
EDC (i.e., CollaMend™ & noncommercial materials)	Increase of enzymatic resistance Upregulation of proinflammatory and angiogenic factors/cytokines Induction of rounded macrophage morphology and macrophage aggregations that form FBGCs to degrade the scaffolds	22,137,142
DHT (i.e., noncommercial materials)	Increase of enzymatic resistance Induction of rounded macrophage morphology and macrophage aggregations that form FBGCs to degrade the scaffolds	137

DHT, dehydrothermal; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FBGCs, foreign body giant cells; GTA, glutaraldehyde; HMDI, hexamethylene diisocyanate; MMP, matrix metalloproteinase.

may be induced by collagen surface modification as a function of cross-linking method employed.[22] Overall, cross-linking of collagen-based devices has been shown to induce a proinflammatory response: macrophage activation and increase of proinflammatory cytokine release. Despite the significant efforts and the advances in elegant readout systems, the mechanism by which cross-linking alters inflammation has not been elucidated as yet.

In Vivo Models for Assessing Host Response to Collagen-Based Devices

In vivo studies assessing host response can be roughly grouped based on the animal model employed (Table 2). Small animal models are primarily utilized to assess inflammatory response to novel devices, while large animal models are used as close replicates of clinical setting. The most common small animal model for collagen-based devices *in vivo* characterization is the subcutaneous implantation in mouse or rat for up to a month in duration.[21,143] A rat full-thickness skin defect model has also been used to evaluate the wound healing ability of collagen materials combined with plastic dressings in acute and chronic wounds.[144,145] The rabbit ear model has also been used to study specific wounds, such as burns[146] and hypertrophic scarring.[147] Inflammatory response and wound healing are evaluated by routine histological analysis that is sometimes complemented with immunostaining and evaluation of protein and gene expression levels. Collagen-based devices have been extensively assessed in large animal abdominal muscle model repair with significant differences in the size of the defect, time points, and characterization methods (Table 2). However, the rat abdominal model has also been used to evaluate collagen devices for soft tissue repair, despite the lower biomechanical stimulus of small animal models compared to large animals. Obviously, the type of the defect depends on the size of the animal; partial-thickness defect is induced in small animals,[148] while full-thickness defect is used in large animals.[149] Furthermore, small animal models are primarily used to study early host response; thus, such studies have more early time points (before 30 days). In contrary, large animal models are primarily focused on long-term response, and therefore, early time points (less than 30 days) are hardly ever of interest. Although routine histological analysis is carried out in both small and large animal models, small animal models use more immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) assays to study inflammation cells, surface markers, proteins, and cytokines, while large animal models study functional parameters, such as histomorphometry and mechanical properties of the new tissue. This deviation may be due to the lack of antibodies for large animal models, such as pig, sheep, and bovine species.

In Vivo Assessment of Host Response to Collagen-Based Devices

Noncross-linked (Table 3) acellular ECM tissue grafts have shown different host responses depending on their origin. Commercially available porcine small intestinal submucosa (SIS, Surgisis™ Soft Tissue Graft; Cook, denaturation temperature of 61–62°C[138,139]) and porcine bladder commercially available (MatriStem; Acell)[20] or research grade[143,150] promoted a dense mononuclear cell infiltration, predominantly neutrophils at week 1 and macrophages (more M2 macrophages than M1) at week 2. At week 4–5, SIS and bladder grafts were completely degraded and were totally replaced by organized collagenous connective tissue and skeletal muscle tissue. Evidence of FBGCs and peri-implantation fibrosis was not observed[20,143] or the fibrous tissue surrounding the implants was $< 52\,\mu m$.[150] In the same way, commercially available human, porcine, and bovine dermis (Alloderm® Tissue Matrix, LifeCell, denaturation temperature 64°C; Strattice™ Reconstructive Tissue Matrix, LifeCell, denaturation temperature 60°C; SurgiMend™ Collagen Matrix for Soft Tissue Reconstruction, TEI Biosciences, denaturation temperature 57°C[139]) demonstrated a dense mononuclear cell infiltration, higher M2 macrophage population than M1, and no presence of FBGCs or encapsulation.[20,151–153] However, dermis grafts showed a lower cell infiltration, degradation, and new tissue formation than those of SIS and bladder,[20,151–153] prolonging graft remodeling over 12 months.[152,153] This longer degradation may be due to the higher organization and density of dermis compared to SIS or bladder. With regard to reconstituted collagen materials, noncross-linked materials have been shown to be well-tolerated, to promote tissue regeneration with a minimal inflammatory response.[154] Finally, the wound healing capacity (cell infiltration, new tissue deposition, and neovascularization) of noncross-linked collagen materials has also been confirmed in the clinical setting.[155]

Chemically cross-linked collagen-based devices demonstrated extended support on the defect area overtime, when compared to the noncross-linked counterparts.[156] Commercially available GTA cross-linked tissue grafts (Table 3) (Peri-Guard® Repair Patch, Synovis; denaturation temperature 83°C[139]) have been shown to elicit chronic inflammation and typical foreign reaction, as evidenced by the early dense accumulation of mononuclear cells and the prolonged presence of macrophages, FBGCs, and fibrous encapsulation surrounding the implant.[149,157,158] GTA cross-linked bovine pericardium grafts reduced the M2/M1 macrophage ratio during the inflammation phase, compared to noncross-linked grafts.[159] Moreover, noncommercial GTA cross-linked sheep collagen disks induced a massive infiltration of neutrophils that secreted a high amount of MIP-1, MCP-1, and IFN-γ, recruiting and activating macrophages. As a result, macrophages upregulated IL-6 and downregulated IL-10, IL-13, promoting FBGC formation.[21] Additionally, commercially available GTA cross-linked collagen sponges for guided bone regeneration and guided tissue regeneration (BioMend® Extend™; Zimmer Dental) promoted ossification *in vivo*; however, the incidence of mucosa tissue perforation was increased.[160] This tissue perforation may be related to the prolonged degradation over 24 weeks, decreased tissue integration and vascularization, and prolonged presence of macrophages and FBGCs around the material.[161] Similar to tissue grafts, noncommercial GTA cross-linked collagen hydrogels demonstrated a reduced *in vivo* degradation (20% degradation after 6 weeks), while their noncross-linked counterparts were largely degraded within a week. However, GTA cross-linked hydrogels reduced cell infiltration and promoted a dense connective tissue layer with inflammatory cells around the

Table 2. Overview of the Animal Models and Experimental Parameters Used to Study Host Response and Wound Healing Associated with Cross-Linked Collagen-Based Materials

Model size	*Species*	*Material*	*Model*	*Time points*	*Characterization*	*References*
Small animal models	C57BL/6 mouse	Dermal sheep collagen disk (GTA, HMDI)	Subcutaneous implantation	2, 7, 14, and 21 days	Toluidine blue staining, PCR (IL-4, IL-6, IL-10, IL-13, IFN-γ, TGF-β, MCP-1, MIP-1), IHC (IL-10), *in situ* zymography	21,168
	Sprague-Dawley rat	Porcine bladder (EDC, GTA), porcine heart valve (GTA), bovine pericardium (GTA)	Subcutaneous implantation	7, 28, 63, and 180 days	HE and MT staining, quantitative stereological analysis	143,150
	Sprague-Dawley rat	SIS (EDC, noncross-linked), porcine dermis (EDC, unknown, noncross-linked),	Partial-thickness defect in abdominal muscle	7, 14, 28, 112 days	MT staining and IHC (CD68, CD80, CD163, CCR7)	20,148
	Wistar rat	Bovine pericardium (GTA, noncross-linked)	Partial-thickness defect in abdominal muscle	21 and 90 days	HE and MT staining, IHC (CD80, CD163), lymphocyte transformation test	159
	Sprague-Dawley rat	Porcine dermis (EDC, HMDI) and human dermis (noncross-linked)	Partial-thickness defect in abdominal muscle	3, 7, 14, 30, 90, and 180 days	HE, MT, and Verhoeff's staining, IF (COLI, COLIII, FN, elastase, and MMP-9)	151
	Sprague-Dawley rat	Collagen hydrogel with or without growth factors	Full-thickness skin defect	3, 7, and 14 days	HE staining, IHC (CD68, COLIV), quantitative stereological analysis, wound contraction	144,145
Large animal models	New Zealand white rabbit	Porcine dermis (EDC, HMDI) and SIS (noncross-linked)	Partial-thickness defect in abdominal oblique muscle	14, 30, 90, and 180 days	MT staining, IF, and qRT-PCR for COLI and COLIII, tensile test	165,166
	New Zealand white rabbit	Collagen sponge with or without growth factors	Second-degree burn defect	15 days	HE staining, tensile test	146
	Yucatan minipig	Porcine dermis (EDC, HMDI), bovine pericardium (noncross-linked), and human dermis (noncross-linked)	Full-thickness defect in abdominal muscle	30, 180, and 360 days	HE staining, stereological analysis, tensile test	149,158
	Caribbean vervet monkey	Porcine dermis (EDC, HMDI) and SIS (noncross-linked)	Full-thickness defect in abdominal muscle	180 days	HE staining, IHC (CD3, CD20, CD68), ELISA (immunoglobulin G and M), tensile test	138,167

COL I, collagen type I; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin and eosin; IF, immunofluorescence; IFN-γ, interferon gamma; IHC, immunohistochemistry; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein-1; MT, Masson's trichrome; PCR, polymerase chain reaction; SIS, small intestinal submucosa; TGF-β, transforming growth factor beta.

Table 3. *In Vivo* Response Associated with Cross-Linked Collagen-Based Materials

Cross-linking agent	*Summary of* in vivo *results*	*References*
Noncross-linked	In general, noncross-linked collagen materials Relevant initial cell infiltration High-ratio M2/M1 macrophages Functional reconstruction, no encapsulation	20,155,158,169,174
	Porcine SIS and bladder (i.e., Surgisis™, MatriStem™, or other noncommercial materials) Fast degradation and remodeling ratio	143,148,150
	Human, porcine, and bovine dermis (i.e., Strattice™ or other noncommercial materials) Lower cell infiltration and over-extended remodeling over 12 months	20,151,153
GTA	Heavily cross-linked collagen-based materials (i.e., Peri-Guard & noncommercial materials) Reduced cell infiltration Low-ratio M2/M1 macrophages Upregulation of proinflammatory cytokines Foreign body response—fibrous encapsulation	21,143,149,150,157–159,162,163
HMDI	Slightly cross-linked collagen-based materials (i.e., Permacol) Similar early recruitment of mononuclear cells than its noncross-linked counterpart and less than noncross-linked SIS and bladder. Reduced cell infiltration Low degradation ratio, over 12–24 months (similar to noncross-linked counterparts) Prolonged presence of macrophages around scaffold Prolonged remodeling and tissue support	23,151–153,155,164–167,170,171,174
	Heavily cross-linked collagen-based materials (i.e., noncommercial materials) Negligible cell infiltration Low-ratio M2/M1 macrophages Limited scaffold degradation over 2 years Upregulation of IL-10 from FBGCs Over-prolonged presence of macrophages and FBGCs around scaffold Chronic inflammation and fibrous encapsulation	21,62,168
EDC	Slightly cross-linked collagen-based materials (i.e., noncommercial materials) Relevant initial cell infiltration Scaffold degradation and remodeling over 180 days New connective tissue replaces degraded scaffold	143,177–180
	Moderately and heavily cross-linked collagen-based materials (i.e., CollaMend & noncommercial materials) Reduced cell infiltration and low-ratio M2/M1 macrophages Limited scaffold degradation over 12 months Prolonged presence of macrophages and FBGCs around scaffold Chronic inflammation and fibrous encapsulation	20,138,148,151,155,165,166,174
Genipin	Slightly cross-linked collagen-based materials (i.e., noncommercial materials) Moderate initial cell infiltration Scaffold degradation and remodeling up to 12 months New connective tissue replaces degraded scaffold	182
	Heavily cross-linked collagen-based materials (i.e., noncommercial materials) Reduced cell infiltration Limited scaffold degradation at 12 months Prolonged presence of macrophages and FBGCs around scaffold Chronic inflammation	181,182

The degree of cross-linking regulates scaffold stability and host response.

hydrogel at an early stage.[162] The nonhealing result of GTA cross-linked collagen materials has been attributed to the toxicity of GTA residues[163] and the high cross-linking density that prohibit degradation and cell infiltration, even after 2 years of implantation.[157]

The slightly cross-linked (HMDI) porcine dermis Permacol (Table 3) has displayed high resistance to degradation *in vivo* and in clinical applications, maintaining the structural integrity for over 2 years.[151,164] Permacol has also been shown to induce early recruitment of mononuclear cells around the graft and limited cell infiltration than its noncross-linked counterpart.[153] However, this early inflammatory cell population recruitment has been shown to be lower for Permacol than noncross-linked SIS and porcine and human dermis (Surgisis, Strattice, and Alloderm, respectively). However, this response was normalized between all grafts over extended periods.[153,165] Regarding cell infiltration, mononuclear cells were detected around Permacol and only infiltrated through material pores; only 20% of implants were colonized at day 14 and the totality of graft was colonized after 1 month.[152,165,166] At 90 days, Permacol showed a higher amount of macrophages (RAM-11 positive, specific antibody for rabbit macrophages) and FBGCs than the noncross-linked SIS graft (Surgisis), but a slightly lower macrophage recruitment than other cross-linked porcine dermis grafts (CollaMend).[166] After 90 and 180 days of implantation, Permacol implants were surrounded by a new randomly organized connective tissue and fibroblast, supporting tissue integration in its immediate environment. This new tissue adhered to the implants, penetrated them through surface pores, and showed a lower collagen density compared with the typical fibrous tissue.[151,165,167] Although Permacol demonstrated a reduction of remodeling ratio, as noncross-linked Strattice and Alloderm, the absence of encapsulation may indicate that these materials are well tolerated and integrated, as they may be assimilated as a normal host matter.

Noncommercial heavily cross-linked (HMDI) dermal grafts (denaturation temperature of 74°C) exhibited extended degradation resistance, induced a limited cell infiltration, and demonstrated a prolonged delay in wound healing.[62] These observations may be related to the IL-10 upregulation from FBGCs, which is known to upregulate transcription of TIMP-1, preventing degradation by MMPs. This has also been observed in heavily cross-linked (HMDI) dermal sheep collagen disks[21] and heavily cross-linked (HMDI) bovine collagen type I disks.[168] The decellularization and delipidation process can also dramatically influence the inflammatory profile of collagen grafts. Specifically, HMDI cross-linked porcine dermis grafts, which were decellularized and delipidated with sodium dodecyl sulfate (SDS) and noncross-linked SIS (Surgisis), have shown increased ROS expression compared to noncross-linked collagen grafts (Strattice and Alloderm™), Permacol, and noncross-linked Permacol.[23] This suggests that ROS increase may be processing-dependent. Finally, Permacol has been widely used for human hernia repair with favorable outcomes, compared to synthetic implants.[169] Nonetheless, tissue grafts have been associated with a 10% failure rate and 14% chronic inflammation issues for the most complex surgery cases for which no ideal material exists as yet.[164,170,171] Commercially available HMDI cross-linked porcine dermis (Zimmer® Collagen Repair Patch; Zimmer) has been used for rotator cuff repair with significant improvement of tendon functionality[172]; however, chronic inflammation has been reported in few cases.[173] It is worth pointing out that these clinical studies were focused on visual observations of the wound and CT scans to evaluate seroma formation, hernia recurrence, and infection. The lack of systematic tissue analysis prohibits precise identification of the cause; is it the graft itself or the comorbidity of the patients?

EDC has also been studied extensively with *in vivo* degradation and host response depending on the tissue graft characteristics (Table 3). Commercially available EDC cross-linked porcine dermis grafts (CollaMend) showed high resistance to degradation, with no degradation signs and no significant cell infiltration for over 180 days.[165] Further, CollaMend induced a disorganized connective tissue with a large amount of macrophages and FBGCs at the implant interface at day 7, reaching the highest cell amount by day 14. By day 30–35, these materials were encapsulated within a dense collagenous tissue and FBGCs[20,151]; encapsulation and a nonconstructive remodeling layer were evidenced over 180 days, the longest published time point.[138,165] Regarding macrophage polarization, CollaMend implants presented the lowest population of M2 macrophages ($CD206^+$) and the highest of M1 macrophages ($CCR7^+$).[20] EDC cross-linked porcine dermis has been employed for human abdominal wall reconstruction and clinical data showed similar recurrence to HMDI cross-linked porcine dermis, largely attributed to poor tissue integration and delay in wound healing.[155,174,175] However, these clinical studies did not assess the inflammatory response in detail. Another commercially available EDC cross-linked SIS (CuffPatch™, Arthrotek) showed similar results to CollaMend; a higher amount of M1 macrophages ($CD80^+$ and $CCR7^+$) than M2 macrophages ($CD163^+$) and a prolonged presence of macrophages and FBGCs over 16 weeks were reported.[148] Interestingly, further investigations demonstrated that the degree of EDC cross-linking of noncommercial decellularized porcine bladder modulated the degradation rate, while it delayed the different stages of reconstructive wound healing.[143] Specifically, the low-dose EDC cross-linked tissue grafts (0.0005 mmol per mg of tissue) were completely infiltrated with host cells by day 7 and remained intact, with new collagen being deposited after 28 days. The degradation of these tissue grafts and new collagenous connective tissue deposition was evidenced up to 180 days. The high-dose EDC cross-linked porcine bladder (0.0033 mmol per mg of tissue) displayed the same tendency than the low-dose EDC with some delays in remodeling: low degradation at day 63 and partial degradation with new organized connective tissue by day 180. Furthermore, the observed outstanding cellular infiltration and remodeling features were attributed to the slight cross-linking degree and the fibroporous structure of the materials.[143] The introduction of interconnected porosity (30–40 μm pore size) has been demonstrated to promote M2 macrophages and to increase material integration.[176] Although scaffold porosity is an important designing parameter, how it modulates macrophage host response is still understudied.

The same host response tendency to EDC cross-linked tissue grafts was observed for EDC cross-linked collagen–

elastin sponges (noncommercial materials)[177]; the low degree of EDC cross-linking (0.3 mM EDC) increased stability of the scaffolds and supported tissue regeneration, although it delayed the wound healing phases. In contrast, the medium degree of EDC cross-linking (0.5 mM EDC) impaired wound healing, induced more macrophages and FBGCs, and scarring was evidenced.[177] Furthermore, low-dose EDC cross-linked collagen conduits and sponges (noncommercial materials) have been shown to increase guidance of regenerating axons through distal peripheral nerve sections without obvious macroscopic signs of inflammation or neuroma formation.[178–180]

As with other cross-linking methods, genipin cross-linking (Table 3) has been shown to increase resistance to degradation for over a year of noncommercial collagen materials, which delayed wound healing.[181,182] 0.00625%, 0.05%, and 0.625% genipin was used to cross-link bovine pericardium tissue grafts. 0.00625% genipin cross-linked grafts were unable to elicit tissue regeneration due to premature degradation and lack of cell support. 0.05% genipin cross-linked grafts promoted a dense layer of inflammatory cells surrounding the grafts and low cell infiltration at day 3. Cell ingrowth increased with time, reaching maximum by month 3. A gradual graft degradation and new tissue deposition were observed over time; the graft was totally degraded and replaced by connective tissue after 12 months. In contrast, 0.625% genipin cross-linked grafts presented more inflammatory cells, less graft degradation, and less tissue replacement; limited graft surface degradation was observed even after 12 months.[182] Ribose has also been used commercially to cross-link collagen sponges (Ossix®; ColBar LifeScience) for guided bone regeneration. This material has demonstrated prolonged degradation, limited cell integration and vascularization, and to induce the presence of macrophages and FBGCs around the material for 24 weeks.[161]

Overall, *in vivo* studies demonstrate that host response depends on the cross-linking density and methods employed. Indeed, slightly cross-linked with HMDI, EDC or genipin collagen-based materials support initial cell infiltration and ultimately scaffold replacement by new tissue. Nonetheless, delays in wound healing have also been reported. On the other hand, heavily cross-linked collagen-based materials promote a proinflammatory response (macrophage activation, predominant M1 macrophage population, and increase of proinflammatory cytokine release) that results in impaired wounds or fibrous encapsulation. Despite the extensive investigation into alternative cross-linking methods, no host response studies have been reported as yet.

Conclusions

To date, there is no gold standard method for cross-linking collagen-based materials. With respect to inflammatory response and wound healing, the vast majority of cross-linking methods remain insufficiently studied, with only GTA, HMDI, and EDC to be the most extensively investigated agents. *In vitro* and *in vivo* data demonstrate that such chemical cross-linking methods alter the normal wound healing process, even at a low concentration. High cross-linking densities are associated with M1 macrophage response and inhibition of M2 macrophage polarization, reduced cell infiltration, increased proinflammatory cytokine expression, chronic wounds, peri-implantation fibrosis, and delayed wound healing. Detailed information of the processing parameters should be provided to enable better appreciation of the device. Furthermore, preclinical and clinical studies should be accompanied with more detailed analysis (e.g., genes, proteins, immunolabeling, and histology) and information of the general physical state of the subject to enable comparison between different studies. We anticipate that an improved understanding of the mechanisms behind the inflammatory and wound healing response to the cross-linkers, coupled with refined experiments and advances in chemistry, will lead to development of alternative cross-linking processes for collagen-based materials that will display an adequate balance of stability, inflammation, and remodeling. However, with currently available chemical agents, mild collagen crosslinking with better processing to eliminate unreacted site products is recommended in complex clinical cases, where resilience to mechanical loading or resistance to enzymatic degradation are prerequisites.

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Disclosure Statement

L.M.D., A.P., and D.Z. declare no competing financial interests. Y.B. is an employee of Covidien - Sofradim Production.

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