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The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development

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Collagen is the oldest and most abundant extracellular matrix protein that has found many applications in food, cosmetic, pharmaceutical, and biomedical industries. First, an overview of the family of collagens and their respective structures, conformation, and biosynthesis is provided. The advances and shortfalls of various collagen preparations (e.g., mammalian/marine extracted collagen, cell-produced collagens, recombinant collagens, and collagen-like peptides) and crosslinking technologies (e.g., chemical, physical, and biological) are then critically discussed. Subsequently, an array of structural, thermal, mechanical, biochemical, and biological assays is examined, which are developed to analyze and characterize collagenous structures. Lastly, a comprehensive review is provided on how advances in engineering, chemistry, and biology have enabled the development of bioactive, 3D structures (e.g., tissue grafts, biomaterials, cell-assembled tissue equivalents) that closely imitate native supramolecular assemblies and have the capacity to deliver in a localized and sustained manner viable cell populations and/or bioactive/therapeutic molecules. Clearly, collagens have a long history in both evolution and biotechnology and continue to offer both challenges and exciting opportunities in regenerative medicine as nature's biomaterial of choice.

1. Introduction

The term "collagen" derives from the Greek words for "glue" and "to produce" and as such it was first known as the component of tissues that when boiled produces glue. The word "collagen" was coined in the 19th century to designate the constituent of connective tissues that yields gelatin after boiling.[1] It has also been considered as the biological glue that holds cells in place.[2] The more modern view is that collagen is the major extracellular matrix (ECM) molecule that self-assembles into cross-striated fibrils, provides support for cell growth, and is responsible for the mechanical resilience of connective tissues.

The prevalence of collagen in human tissues and various inherent properties (e.g., cell recognition signals, ability to form 3D scaffolds of various physical conformations, controllable mechanical properties, and biodegradability) makes it a natural choice as raw material for tissue-engineered scaffolds for various clinical indications. The desirability of collagen as a biomaterial principally depends on the fact that it is a naturally abundant extracellular matrix component and, as such, it is perceived as an endogenous constituent of the body and not as foreign matter.

Collagen is a complex supramolecular structure and occurs in highly diverse morphologies across different tissues, thus

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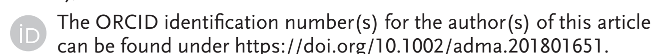
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lending them a range of biological functions. Collagen components interact sequentially with each other and with other ECM constituents to produce higher order structures with numerous hierarchical levels of association and specific functions. Further, collagen, as the fundamental structural component of connective tissues, plays a pivotal role in maintaining their structural and biological integrity. Advanced understanding of these properties has paved the path for the development of novel biomaterials that mimic both the structural and biological properties of native tissues, particularly tissues primarily comprised of collagen type I or collagen type II.

To fully exploit the potential of this unique biopolymer in biomedicine, it is essential to understand its fundamental characteristics, key processing modes, and application features. To this end, we provide an overview of the suprafamily of collagens and their biosynthesis, assembly, and native crosslinking. We also critically discuss current various sources of collagen, natural to synthetic, along with collagen-based device fabrication, crosslinking, and characterization methods. We further highlight significant new knowledge on collagen as a biopolymer that will effectively drive innovation in reparative therapies in the years to come.

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2. Collagen Family

The collective term "collagen" encapsulates a whole family of glycoproteins that are characterized by three signature features. First among these is the amino acid repeating sequence $[Gly–X–Y]_n$, both with and without interruptions. The second characteristic feature is the occupation of the X and Y positions by proline and its hydroxylated form, hydroxyproline, respectively. Third, the right-handed triple helix is formed from three left-handed polyproline α-chains of identical length, which gives collagen a unique quaternary structure.

The ubiquity of collagen and collagenous structures throughout the animal kingdom serves as an indication of their importance in biological viability. Sponges, the simplest known multicellular organisms, express genes for at least two types of a prototypic collagen.[3] In vertebrates, collagen is the major component of specialized and nonspecialized connective tissues, making up almost one-fourth of the total body protein in humans, three-fourths of the dry weight of human skin, over 90% of human tendon and corneal tissues, and almost 80% of the organic matter in bones.[4]

It is interesting to note that the triple helical blueprint has been partially carried over into the structures of other complex molecules that have evolved in air-breathing animals with advanced immune and nervous systems. A data bank search (Source: www.uniprot.org; term searched: collagen-like domains; species: human) yielded 42 glycoproteins that are not bona fide collagens. These include all three subunits of complement component C1q, 13 proteins related to them, as well as adiponectin, collectins, EMILINs, gliomedin, neurogranin, otolin-1, macrophage scavenger receptors, mannose-binding protein, pulmonary surfactant proteins A1/A2 and D, and the collagenic tail peptide associated with acetyl cholinesterase.

Evolutionary branching, partially by reduplication of chromosome parts, has led to a multitude of genetically distinct collagen types; 29 have been described to date.[5] Notably, these collagen types were discovered through their homologies to other collagen genes and their characteristic $[Gly–X–Y]_n$ sequences. Although the tissue distribution and function of many collagen types still remain obscure, along with confirmation of their existence on the protein level, it is clear that collagens occur in many places throughout the body, with collagen types I, II, and III representing the lion's share; together they make up around 80–90% of the total body collagen.

2.1. Collagen Structure and Conformation

The collagen molecule is comprised of a triple helical region and two nonhelical regions at either end of the helix. The triple helical conformation is the defining structural element of all collagens (**Figure 1**a). The collagen triple helix (tertiary structure) has a coiled-coil structure made of three parallel α polypeptide chains (secondary structure) that are wound around each other in a regular helix to generate a rope-like structure of ≈300 000 g mole^{-1} molecular weight and 280 nm in length and 1.4 nm in diameter. Intramolecular hydrogen bonds between glycines in adjacent chains stabilize the triple helix. The hydroxyl groups of hydroxyproline residues also form hydrogen bonds and stabilize the triple helix. Two hydrogen bonds per triplet are found: one between the amine group of a glycyl residue and the carboxyl group of the residue in the second position of the triplet

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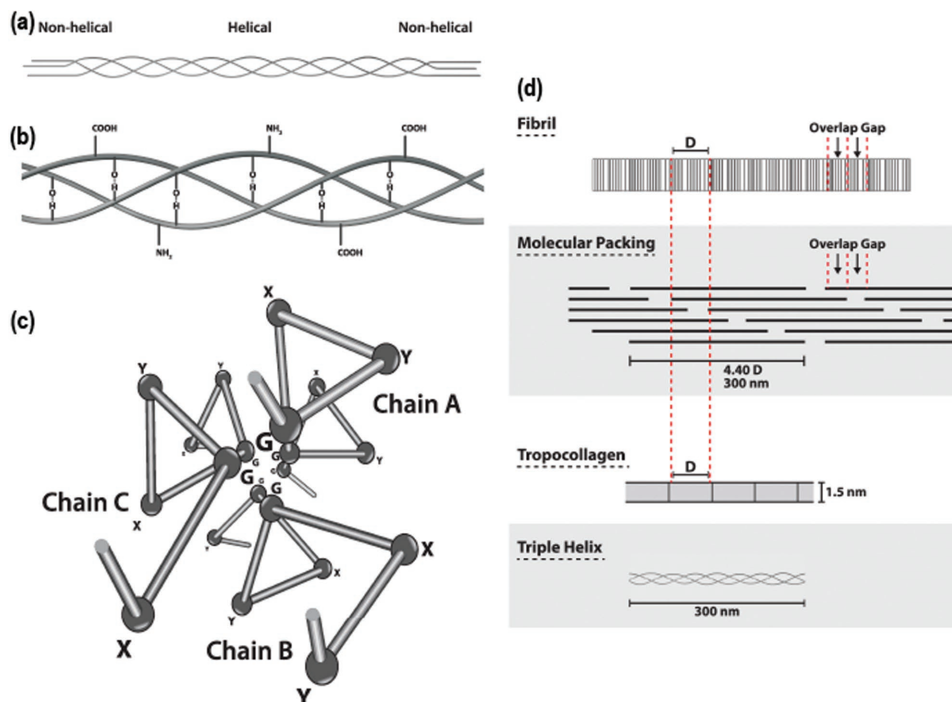

Figure 1. a) The triple-helical collagen structure. b) Hydrogen bonds within the collagen triple helix. c) Cross-section of collagen triple helix. d) Schematic representation of the arrangement of collagen molecules within fibrils.

in the adjacent chain, and one via the water molecule participating in the formation of additional hydrogen bonds with the help of the hydroxyl group of hydroxyproline in the third position (Figure 1b). Each α-chain is left-handed, but when they are staggered by one residue relative to each other around a central axis, they form a right-handed superhelix (Figure 1c). This superhelix is due to the twisting of the chain helices around the central axis by about +30° at every turn. Thus, every third amino acid is in the center of the helix and, for steric reasons, only glycine, with a side chain limited to a single hydrogen atom, can occupy this position without altering the triple helical conformation. The Gly–Pro–Hyp sequence is the most common (about 12%), sequences of the form Gly–Pro–Y and Gly–X–Hyp represent about 44% and Gly–X–Y sequences constitute the remaining 44%. Proline and hydroxyproline stabilize the collagen molecule because of their alicyclic nature, and they stiffen the α-chain, where they occur by preventing rotation around the C—N bond. During or following secretion in the extracellular space, the propeptides are removed at either end of the triple helical molecule by specialized enzymes, leaving the triple helix with short, nontriple helical regions, measuring 9–26 amino acids in length at the N- and C-termini. These nonhelical domains, referred to as telo-peptides, play a crucial role in the registering (the alignment of the three pro-α-chains) and crosslinking of collagen α-chains and they also add flexibility to the otherwise rigid molecule. The removal of the propeptides is prerequisite to the self-assembly of collagen molecules into a quarter-staggered arrangement by lateral and head-to-tail fashion, ultimately resulting in the formation of cross-striated fibrils.

Collagen type I, the most abundant collagen type, is present in the form of elongated fibrils that can be greater than 500 µm in length, 500 nm in diameter, and contain more than 10^7 molecules. The collagen fibrils exhibit a high degree of axial alignment, which results in a characteristic D banding/periodicity, due to the alternating overlap (two adjacent triple helices) and gap (triple helices lined up head to tail with some space in between) zones, produced by the specific packing arrangement of the 300 nm long and 1.5 nm in diameter collagen molecules. This produces an average periodicity of 67 nm in the native hydrated state (Figure 1d) although dehydration and shrinkage during conventional sample preparation for electron microscopy results in lower values of around 55–65 nm. The in vitro fibrillogenesis of collagen type I is dependent on temperature, pH, and ionic strength. Under appropriate conditions, collagen molecules will spontaneously self-assemble to form microscopic fibrils, fibril bundles, and macroscopic fibers that exhibit D periodicity banding virtually indistinguishable from native collagen fibers. This feature was first described in the late 1940s with transmission electron microscopy (TEM)[6] and is absolutely typical of collagen. Topographical analysis of the surface of large collagen fibers by atomic force microscopy (AFM) confirmed ridges alternating with 5–15 nm deep grooves with a 60–70 nm period.[7]

2.2. Collagen Types

To date, 40 vertebrate collagen genes have been identified that form 29 distinct homo- and/or heterotrimeric molecules (**Table 1**).[8,9] Roman numerals are used to indicate the type and Greek letters are used to identify the chains, bands, and

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Table 1. Collagen family characteristics and tissue distribution (adopted with modifications from ref. [9]).

Collagen type	Chains	Molecular assembly	Supramolecular structure	Mw [kDa]/α-chain	Tissue distribution
I (Heterotrimer)	$[\alpha1(\mathrm{I})]_2\alpha2(\mathrm{I})]$	Monomers staggered by 67 nm	Large diameter, 67 nm banded fibrils	95	Skin, tendon, ligament, cornea, organ capsules, dura mater of brain and spinal cord, the main organic component of bone
I (Homotrimer)	$[\alpha1(\mathrm{I})]_3$		67 nm banded fibrils		Tumors, dermis, bone
II	$[\alpha1(\mathrm{II})]_3$	Monomers staggered by 67 nm	67 nm banded fibrils	95	Cartilage, vitreous, cartilagenous zones of tendon, intervertebral disc
III	$[\alpha1(\mathrm{III})]_3$	Monomers staggered by 67 nm	Small diameter, 67 nm banded fibrils	95	Dermis, aorta, uterus, admixture in tendon, intestine, blood vessels, in the reticular connective tissue of liver, spleen and surrounding internal organs
IV	$[\alpha1(\mathrm{IV})_2\alpha2(\mathrm{IV})]$; $\alpha3(\mathrm{IV})$, $\alpha4(\mathrm{IV})$, $\alpha5(\mathrm{IV})$, $\alpha\,6(\mathrm{IV})$	Association of 4N- and 2C-termini	Nonfibrillar meshwork	170–180	Basement membranes
V	$[\alpha1(\mathrm{V})]_2\alpha2(\mathrm{V})$ $[\alpha1(\mathrm{V})\ \alpha2(\mathrm{V})$ $\alpha3(\mathrm{V})]$ $[\alpha1(\mathrm{V})]_3$	Monomers staggered by 67 nm	9 nm diameter banded fibrils	120–145	Placental/embryonic tissue, dermis, bone, cornea, cell surfaces
VI	$[\alpha1(\mathrm{VI})\ \alpha2(\mathrm{VI})\ \alpha\alpha3(\mathrm{VI})]$	Association into tetramers that aggregate end to end	5–10 nm diameter	$\alpha l(\mathrm{VI})$ 140	Uterus, dermis, cartilage
			beaded microfibrils	$\alpha2(\mathrm{VI})$ 140	Muscle
			100 nm periodicity	$\alpha(\mathrm{VI})$ 340	
VII	$[\alpha1(\mathrm{VII})]_3$	Lateral aggregation of antiparallel dimers	Anchoring fibrils	170	Skin, amniotic membrane, cornea, mucosal epithelium
VIII	$[\alpha1(\mathrm{VIII})]_2$ $\alpha2(\mathrm{VIII})$	Interrupted helical structure	Nonfibrillar, hexagonal lattice	61	Descemet's membrane, endothelial cells
IX	$[\alpha1(\mathrm{IX})\ \alpha\,2(\mathrm{IX})$ $\alpha\alpha3(\mathrm{IX})]$	Covalently crosslinked to surface of collagen II fibrils	FACIT; nonfibrillar	68–115	Cartilage, vitreous, admixture in tendon, codistributes with collagen II
X	$[\alpha1(\mathrm{X})]_3$	Assemble a mat-like structure	Nonfibrillar, hexagonal lattice	59	Calcifying cartilage (including parts of tendons)
XI	$[\alpha1(\mathrm{XI})\ \alpha\,2(\mathrm{XI})\ \alpha\alpha3(\mathrm{XI})]$	Monomers staggered by 67 nm	Fine fibrils similar to those of collagen V	110–145	Cartilage, intervertebral disc
XII	$[\alpha\,1(\mathrm{XII})]_3$	Associates with surface of collagen fibrils	FACIT; nonfibrillar	220, 340	Dermis, tendon, cartilage
XIII	$[\alpha1(\mathrm{XIII})]_3$	150 nm rod with two flexible hinges	Transmembrane	62–67	Endothelial cells, epidermis
XIV	$[\alpha1(\mathrm{XIV})]_3$	Disulfide-linked cross-shape	FACIT; nonfibrillar	220	Dermis, tendon, cartilage
XV	$[\alpha1(\mathrm{XV})]_3$	Figure eight knot configuration	MULTIPLEXIN; nonfibrillar	125	Placenta, kidney, heart, ovary, testis
XVI	$[\alpha1(\mathrm{XVI})]_3$	Associates with dermal fibrillin; associates with banded collagen in cartilage	FACIT; nonfibrillar	150–160	Heart, kidney, muscle
XVII	$[\alpha1\ (\mathrm{XVII})]_3$	Shed from cell surface into shorter soluble form	Membrane-intercalated	180	Hemidesmosomes (skin), specialized epithelia
XVIII	$[\alpha1(\mathrm{XVIII})]_3$		MULTIPLEXIN; nonfibrillar	200	Kidney, liver
XIX	$[\alpha1(\mathrm{XIX})]_3$	Sharply kinked and higher order complexes	FACIT; nonfibrillar	165	Transitory embryonic expression, interneurons and formation of hippocampal synapses, basement membranes, muscle cell, rhabdomyosarcoma
XX	$[\alpha1(\mathrm{XX})]_3$	Binds to collagen fibrils with amino terminal domains away from fibrillar surface	FACIT	185, 170, and 135	Corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	$[\alpha1(\mathrm{XXI})]_3$		FACIT		Blood vessel walls, secreted by smooth-muscle cells
XXII	$[\alpha1(\mathrm{XXII})]_3$	Associates with cartilage microfibrils	FACIT	200	Tissue junctions
XXIII	$[\alpha1(\mathrm{XXIII})]_3$		Transmembrane		Tumors (prostate)

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Table 1. Continued.

Collagen type	Chains	Molecular assembly	Supramolecular structure	Mw [kDa]/α-chain	Tissue distribution
XXIV	$[\alpha1\,(XXIV)]_3$	Associates with vertebrate fibrillar	Fibrillar, fibril associated		Regulation of collagen I fibrillogenesis, osteoblast differentiation marker
XXV	$[\alpha1\,(XXV)]_3$	Binds to fibrillized Aβ	Transmembrane	50/100	Interaction with β amyloid plaques in Alzheimer's disease
XXVI	$[\alpha1\,(XXVI)]_3$		FACIT	≈80	Ovary and testis
XXVII	$[\alpha1\,(XXVII)]_3$	10 nm network organization	Thin nonstriated fibrils		Hypertrophic cartilage
XXVIII	$[\alpha1\,(XXVIII)]_3$	Associates with nonmyelinated regions	Beaded filament forming	≈50	Basement membrane of Schwann cells, peripheral nervous system
XXIX	$[\alpha1\,(XXIX)]_3$		Nonfibrillar		Suprabasal cells in epidermis, lung, small intestine, colon and testis

higher molecular weight components. The trimeric nature of a collagen molecule allows for the combination of three identical pro-α-chains or of two identical chains and one other with fitting length and registration of C-propeptide, or even of three different chains to form a complete triple helix. The fit of the respective pro-α-chains, as defined by their length, corresponding interruptions (if any), the correct registration of the C-propeptides, and their combination, delineates different collagen types. However, isoforms within individual collagen type do exist. For example, most collagen type I exists as heterotrimer of two $\alpha1$ chains and one $\alpha2$ chain, but also as homotrimer of three $\alpha1$ chains. Many collagen types, such as collagen type II, type III, or type VII, exist exclusively as homotrimers. At the other end of the spectrum is collagen IV, where six different α-chains are available for combination to yield a considerable number of isoforms that form tissue-specific basement membranes.

While some of the 29 currently identified collagen types show highly unique features, most of them appear highly interrelated, but confined to specific tissue locations. This variety points to diverse biological functions reflected by a multitude of physical structures. Based on their primary structure, the length of the triple helical domain, the molecular weight, the charge profile along the helix, the triple helix interruptions, the size and shape of the terminal domains, the cleavage or retention of the latter in the supramolecular aggregate, and variation in the post-translation modifications, four overarching collagen groups can be identified[10]:

Group 1 hosts the fibril-forming collagen type I, type II, type III, type V, type XI, type XXIV, and type XXVII. They all possess triple helices with uninterrupted Gly–X–Y stretches ≈300 nm in length. However, XXI and XXVII show imperfections in these Gly–X–Y stretches, suggesting very short interruptions of triple helical structure. Collagen fibrils in the dermis, tendon, and other tissues are often mixtures of different collagen types, usually type I, type III, and type V. These mixed fibers are referred to as heterotypic fibrils, contrasting with homotypic fibrils that are composed of only one collagen type (e.g., collagen VII in anchoring fibrils of the dermo–epidermal junction).

Group 2 hosts the basement membrane collagen type IV, type VII, and type XXVIII. While collagen type IV forms a fibrillar meshwork, collagen type VII is created through antiparallel dimer association and forms cross-striated fibrils with a different banding pattern.

Group 3 contains the short-chain collagen type VI, type VIII, and type X. They are named after their triple helical regions, which extend up to 100 and 150 nm, respectively. Collagen type VI forms beaded microfilaments, while collagen type VIII and type X form hexagonal lattices. Collagen type XXIX has a short and uninterrupted triple helical region that is flanked by several von Willebrand factor A domains.

Group 4 contains collagens with multiple interruptions of their triple-helical Gly–X–Y stretches. Collagen type IX, type XII, type XIV, type XVI, and types XIX to XXII comprise the fibril-associated collagens with interrupted triple helices (FACIT collagens). These collagens fulfill specific roles by association with collagen fibrils and adding functionality to them. They may also play a role in controlling the diameter of collagen fibers in various tissues by limiting lateral appositional growth, as has been described for collagen type IX,[11] but also for the fibrillar collagen type V.[12] The term MULTIPLEXINs (multiple triple-helix domains and interruptions) has been created for collagen type XV and type XVIII, as they present the highest number of interruptions. A remarkable subgroup of the nonfibrillar collagens is the transmembrane collagens (type XIII, type XVII, type XXIII, and type XXV), which possess transmembrane domains that allow these molecules to be inserted into cell membranes, while projecting the (interrupted) triple-helical domains outward into the extracellular space.

At supramolecular assembly level, admixtures of fibrillar collagen types that lead to heterotypic fibrils are identified. A typical extract of dermis will show a combination of collagen type I, type III, and type V in varying proportions, as will biochemical analysis of matrix that has been deposited by cultured dermal fibroblasts isolated from this tissue. On top of these heterotypic fibrils, nonfibrillar collagens and other ligands, such as proteoglycans, are identified. Major advances have been made in identifying fibrillar composition using highly sensitive techniques, such as infrared matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (IR-MALDI-TOF-MS).[13] Thus, the composition of triple helices, the supramolecular heterogeneity of fibrils, and finally the admixture of nonfibrillar ligands generates the biological versatility and functionality of the collagens.

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3. Collagen Biosynthesis

3.1. Intracellular Events and Triple-Helix Formation

The pathway of collagen biosynthesis, from gene transcription to secretion and aggregation of collagen monomers into functional fibrils, is a complex multistep process, requiring the coordination of numerous temporally and spatially coordinated biochemical events (**Figure 2**). Depending on the collagen type and isoform, the initial step of the intracellular biosynthesis of collagen involves transcription of mRNA molecules encoded by various three-chain combinations of different α-chain genes. The nascent collagen α-chain enters the lumen of the endoplasmic reticulum with the N-terminus first as pre-procollagen, which is converted into procollagen by the removal of the signal peptide. A remarkable feature of collagen biosynthesis is the fact that synthesis starts at the N-terminus, while triple-helix formation starts at the C-terminus.[14] This requires the pro α-chains to remain untangled for the timespan taken to complete the α-chain translation, upon which three pro-α-chains align precisely at the C-terminus before triple-helix formation begins. Several chaperone proteins protect α-chains from getting tangled, including prolyl 4-hydroxylase (P4-H), protein disulfide isomerase (PDI), a homologue of heat shock protein 70 of the endoplasmic reticulum (BiP/Grp78), various peptidyl-prolyl *cis–trans* isomerases (PPIases), and heat shock protein 47 (hsp47).[15]

For collagen type I, the most abundant collagen type, the alignment of the three pro-α-chains is called registration and is driven by the C-telo-peptides. The C-propeptides contain cysteines, which form disulfide bonds (the only covalent bonds in the procollagen trimer) that will disappear with the removal of the propeptides upon secretion. Intracellularly, this allows for a firm alignment, preventing any slippage of α-chains against each other. The triple helical formation then propagates in a zipper-like manner from the C- to the N-terminus.[16] It takes an average of 14 min for a procollagen type I triple helix to fold, a considerable time span for a single molecule. hsp47 has been shown preferentially bind to procollagen after triple helical folding has taken place, attaching to Gly–X–Y repeats with Arg in the Y position and thereby lending stability to the triple helix and preventing the premature aggregation of procollagen.[15,17] However, hsp47 detaches after procollagen transfers to the Golgi apparatus from the endoplasmic reticulum, probably due to pH change. Interestingly, for collagen type I, trimers consisting of [pro α1(I)]3 and [(pro α1(I))2, pro α2(I)] can be formed, but [pro α2(I)]3 trimers have never been retrieved from cell culture or intact tissues.

3.2. Post-translational Modification of Collagens

Two major post-translational modifications (PTMs) of collagen, hydroxylation and glycosylation, occur in the endoplasmic reticulum, which contribute to the thermal and mechanical stability of collagen in triple helical and assembled form, respectively. Pathological conditions that interfere with these PTMs, either by genetic alteration (e.g., Alport Syndrome) or by nutritional deficiencies, have also been reported.

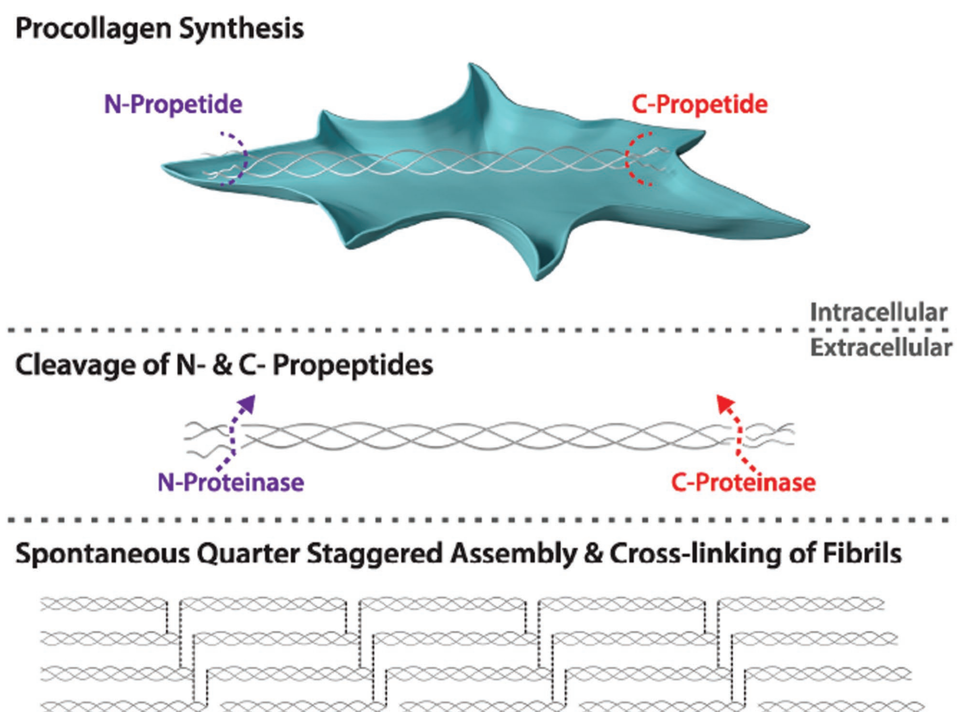

Figure 2. Biosynthesis and processing of collagen. Procollagen is synthesized intracellularly with intact propeptide extensions. Following or during secretion in the extracellular space, specific cleavage of the N- and C-propeptide extensions, by the N- and C-proteinases respectively, takes place. This triggers the spontaneous quarter staggered assembly of collagen into fibrils, which are stabilized through various crosslinking pathways.

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3.2.1. Prolyl Hydroxylation

The signature amino acid of collagen, hydroxyproline (Hyp), is derived from Pro by catalytic activity of prolyl 4-hydroxylase (procollagen–proline dioxygenase; E.C. 1.14.11.2), an enzyme resident in the lumen of the endoplasmic reticulum of fibrogenic cells. Hyp represents ≈10% of the amino acid composition of collagen and is usually present at the Y position in the Gly–X–Y repeat domains. It therefore can be regarded as a molecular fingerprint of collagen. The content of Hyp is critical for the formation of intramolecular H bonds within the triple helix, which in turn confer thermal stability (at body temperature) to the trimer. In mammals with a body temperature of around 37 °C, a minimum of 100 Pro residues per pro-α-chain must be converted to Hyp to achieve thermal stability, while in cold blooded species (e.g., arctic cod) much less hydroxylation is evident.[18] Although this correlation between increased body temperature and Hyp content in multicellular organisms is nonlinear,[19] it points to a remarkable enzymatic flexibility of collagen's thermal stability, which allowed collagens to accommodate different body temperatures across the evolution of fish, amphibians, reptiles and mammals.[20] The role of 3-prolyl hydroxylase has been less clear, but it seems to be associated with modifications of the C-termini of the α-chains of collagen type I and type III. These regions contain stretches of $[GPP]_5$ and $[GPP]_7$, respectively, that are rich in Hyp and appear to particularly increase local thermal stability.[21] These $[GPP]_n$ regions seem to be preferentially modified by 3-prolyl hydroxylase. While the significance of this PTM remains unclear, it is a particular feature of tendon and appears to have contributed to the structural evolution of this connective tissue.[22]

The role of Hyp in stabilizing the triple helix via hydrogen bonds was contested in the late 1990s by studies using synthetic halogen-substituted peptides, like $[ProFlpGly]_{10}$, where Flp was a 4(R)-fluoroproline residue, with Flp being the most electronegative element and incapable of forming H bonds.[23] The resulting hyper-stability in the absence of H bonds and water networks was attributed to exopyrrolidine ring pucker and trans/cis preferences mediated by its electronegative inductive effect; this has led to further investigations of the puckering states of the proline pyrrolidine ring.[24] It is currently debated whether Flp and Hyp stabilize the collagen triple helix in the same way. As an additional explanation for the formation of hyperstable triple helices with halogen-substituted Pro, interstrand dipole–dipole interaction have been proposed to take effect, as compensating forces between electronegative substituents of Pro derivatives in the X and Y positions.[25] Therefore, inductive effects and H bonding of Hyp through hydration networks are now both accepted mechanism of triple helix stabilization.[26]

Interestingly, there are alternatives to prolyl hydroxylation to stabilize collagenous polyproline coils in invertebrates and bacteria. The cuticle collagen of the deep-sea hydrothermal vent worm *Riftia pachyptila* has thermal stability at 37 °C despite a very low Pro content (5%) and therefore a low Hyp content. Thr occupies the Y position of Hyp in the Y position, representing 18% of the total amino acid content and showing O-glycosylation. This PTM is required for the triple helix stability in this species.[27] The cell surface protein Scl2 of *Streptococcus pyogenes* contains a sizeable collagenous domain of 79 Gly–X–Y triplets, resulting in melting temperatures of 36 °C at neutral pH, thus matching human body temperature. The reason for this stability seems to be the relative abundance of Gly–Lys–Asp triplets, contributing to considerable electrical charge; thus allowing for electrostatic interactions between α-chain equivalents, including a hydration network in the absence of Hyp.[28] The new understanding of Hyp-free stability of collagenous domains in bacterial species, which act as a pathogens to mammals, points to a coevolution of stabilizing strategies for polyproline triple helices at mammalian body temperatures and underlines the feasibility of producing and applying bacterial collagens for biomaterial purposes.[29]

3.2.2. Enzymatic Glycosylation and Lysyl Hydroxylation

As a glycoprotein, collagen type I has a relatively low carbohydrate content (<1%). The sugar components in collagen are either a single galactose unit or a disaccharide of galactose and glucose, O-glycosidically attached via hydroxylysine residues. Collagen also contains hydroxylysine (Hyl), a PTM compound of lysine that is produced via lysyl hydroxylase (E.C. 1.14.11.4) activity. The formation of Hyl residues and subsequent attachment of sugar components appears to be an important modulator of fibrillogenesis and is associated with covalent crosslinking and fibril stabilization. O-linked glycosylation of Hyl residues has long been known to be a unique PTM for collagens and proteins with collagenous sequences. It is interesting that human lysyl hydroxylase isoform 3 (LH3) possesses both lysyl hydroxylase and glucosyl transferase (GGT) activities.[30] Transfections studies with LH3 in osteoblast cultures revealed five glycosylation sites in type I collagen, one of them including a major helical crosslinking site. Manipulation of LH3-mediated glycosylation resulted in different collagen crosslinking, fibrillogenesis and mineralization.[31] N-linked glycosylation has been shown to be restricted to propeptide regions of some mammalian collagens[32] and adjoined regions of collagenous domains of some invertebrates.[33] In mammals, potentially *N*-glycosylated regions are lost after the proteolytic conversion of procollagen to collagen.

3.3. Proteolytic Cleavage of Procollagen

The procollagen trimer is released and secreted to the extracellular space, but only if the triple helix is completely folded. The quality-control mechanism measuring triple helicity, along with the sorting mechanism that allocates appropriate α-chains to the respective nascent triple helices in precise stoichiometric relationships, is only partially understood. Heat shock protein 47 is a collagen binding chaperone that assists in stabilizing correctly folded procollagen.[34] Protein disulfide isomerase, a subunit of the prolyl hydroxylation complex, also serves as a chaperone during the assembly of procollagen α-chains[35] and assists in preventing nonassembled procollagen leaving the endoplasmic reticulum.[36] Upon or during secretion into the extracellular space, procollagen is proteolytically processed. Initially, the N- and C-propeptides are removed enzymatically

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in the presence of Ca^{2+} by procollagen N-proteinase and procollagen C-proteinase, respectively. In procollagen type I, procollagen N-proteinase cleaves N-terminal propeptides between Pro and Gln residues, while procollagen C-proteinase cleaves between Ala and Asp. The N-proteinases belong to the ADAMTS (a disintegrin and a metalloproteinase with thrombospondin repeats) family, while procollagen C-proteinases are now classified as bone morphogenetic protein-1/Tolloid-like proteinases (BTPs)[37] and simultaneously trigger matrix assembly and boost the synthesis of matrix proteins via a direct effect on growth factors, such as TGF-β and IGFs.[38]

Another group of metalloproteinases, meprins, are capable of removing both N- and C-terminal collagen propeptides.[39] In line with this are studies showing that removed propeptides can re-enter the cell to regulate the amount of collagen biosynthesis taking place on the basis of a negative feedback loop.[40] The propeptides of intact procollagen prevent premature intracellular supramolecular assembly and formation of water-insoluble aggregates, but need to be removed in the extracellular space to allow collagen assembly. Thus, procollagen proteinase activity is a rate-limiting step for fibrillogenesis.[41]

3.4. Extracellular Supramolecular Assembly

After the enzymatic removal of the propeptides, the resulting collagen triple helices (also described as tropocollagen) are able to form supramolecular aggregates. The debate as to where exactly the procollagen/collagen conversion occurs is not yet settled and two potential models seem plausible with respect to the release of procollagen.[42] The first model proposes that fibril formation begins inside the Golgi-to-plasma membrane carriers (GPCs), where cleavage of procollagen propeptides already occurs, after which GPCs containing newly formed fibrils fuse and form finger-like structures at the cell surface, probably with cytoskeletal contribution. The second model describes collagen fibrillogenesis as a mostly extracellular process, whereby collagen fibril formation occurs at the surface of fibroblasts in deep invaginations of the plasma membranes, where narrow elongated "hangars" formed through the merging of collagen-containing GPCs. Although enveloped partially by the plasma membrane, the interior of these "hangars" is part of the extracellular space. It is here that propeptide removal occurs and after procollagen cleavage of the C- and N-propeptides, the collagen molecules aggregate to form collagen fibril intermediates that grow out of their "hangars." This theory takes into consideration spatial constraints for secreting bulky procollagen molecules, essentially linear rods, via the Golgi apparatus in GPCs.

Fibrillogenesis cannot occur in vivo without the mediation of cells that engage nascent and mature fibrils via cell surface receptors (e.g., integrins). The peptide sequence Arg–Gly–Asp is a significant feature of the glycoprotein fibronectin, representing an integrin-binding site, along with a collagen- and gelatin-binding site. Accordingly, integrins and fibronectin have been described as "fibril organizers", whereby fibronectin forms a fibril network which is then engaged by integrins, thus serving as a template for further collagen fibril assembly.[43,44] Notably, collagen fibrils assembled in vivo and ex vivo (in cell culture) are heterotypic.[45] The admixture of minor collagens (e.g., collagen V and XI) forms the side of dermal collagen type I and cartilage collagen type II fibers, respectively, with N-terminal domains at the fibril surface. This suggests a similar nucleating function with a diameter-limiting effect.[44] As such, collagen fibrillogenesis is affected by cell-fiber contact, by the reshuffling fibers resulting from cell movement, by ligands that control the growth of fibers and by proteolytic enzymes that remodel the deposited matrix. For example, studies of the corneal stroma of the developing chick eye have revealed the intricate assembly of the stromal ECM, which is finely controlled to build the correct shape and transparency. The corneal stroma is characterized by homogeneous collagen fibrils of small diameter, the size of which is controlled by accessory molecules, such as FACIT collagens and small leucine-rich proteoglycans,[46] and shows a highly ordered hierarchical organization.[47]

Molecular packing of collagen molecules then takes place, with certain structural features applying to almost all collagenous fibrillar structures.[48] The fibril-forming collagens are subdivided into type I rich fibrils (containing predominantly collagens II and V) and type II rich fibrils (more typically containing collagen types IX and XI).[48] Collagen triple helices form longitudinal structures by lateral alignment and with a stagger of roughly one fourth of the molecular length. The pairing occurs between a stretch of 234 amino acids of either helix, a region that ensures maximal electrostatic interaction and hydrophobic interactions. The molecular stagger leads in projection to regions of high and low electron density, namely the overlap (two adjacent triple helices) and gap (triple helices lined up head to tail, but with some space between them) regions. The key to further axial growth seems to be the interaction of telo-peptide regions of a triple helix with an adjacent trimer. Current models suggest a hook-like back-folding of C-telo-peptides, bringing Tyr residues within the telo-peptide trimer into axial vicinity, while bringing a Lys residue in a position to register with a Hyl residue in a triple helical domain of an adjacent triple helix.[48] 3D packing of collagen trimers of collagen type I includes five trimers in a pentagonal arrangement forming a microfibril.[49] This popular model has been recently revised to a compressed five-stranded microfibril that forms a trapezoid and accounts for the degree of crystallinity seen in collagen fibers. This model accommodates both crystallinity and liquid-like disorder, suggesting a concentric and appositional packing of these structures, where the gap regions represent disordered areas.[48] The packing of molecules deviates by roughly 5° from the longitudinal axis (molecular tilt) in tendons and by up to 18° in dermis. Subsequent modeling and ultrastructural investigation showed that collagen fibrils pack and grow in a helical fashion, reminiscent of winding techniques used in rope making.[50] The 67 nm axial repeat is the most frequently observed in collagen-containing tissues in animals, although shorter (e.g., 9 and 23 nm) and larger (e.g., 150–250 nm, named fibrous long spacing (FLS)) periods have been reported. The range of diameters of collagen fibers found in mammalian tissues spans 2 orders of magnitude. Thus, the enigma of which factors assign particular diameters to specific tissues and keep them in homogenous distribution over a lifespan remains unanswered. Replenishing of molecules, remodeling of structures and age-related changes has been shown to be critical in this process.[51]

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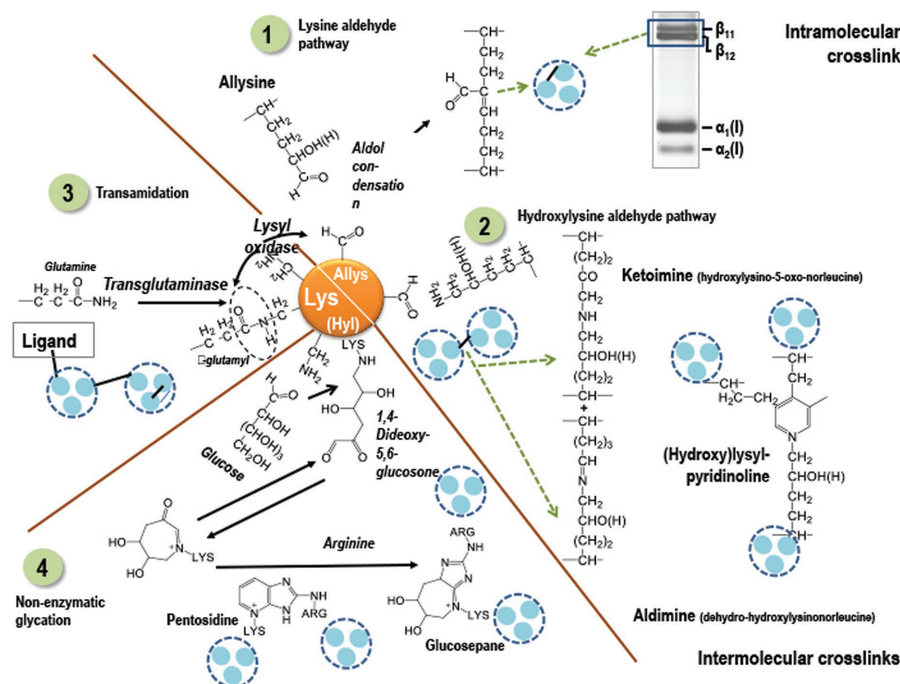

Figure 3. 1) Lysyl oxidase–mediated crosslinking. Lysyl oxidase desamidates lysine to allysine and hydroxylysine to hydroxyallysine (not shown). The lysine aldehyde (allysyine) pathway leads to aldol condensation and intramolecular crosslinks within a given triple helix, which are evident in SDS-PAGE gels (here shown after silver staining). 2) The hydroxylysine pathways leads to ketoimine and aldimine crosslinks, respectively, which bridge two separate collagen triple helices. With a third partner these crosslinks mature to nonreducible hydroxyl pyridinolines. 3) Transglutaminase-mediated isopeptide crosslinks affix mainly noncollagen ligands to collagen, but also form intra/intermolecular collagen crosslinks too. 4) Nonenzymatic glycation. Glucose plays role in the formation of intermolecular crosslinks by forming a Schiff base with Lys, and then an Amadori product. Finally, a ring structure with Arg is formed, resulting in glucosepane; a comparable structure, pentosidine, is formed with ribose.

3.5. Natural Crosslinking

The hierarchical assembly/packing of collagen molecules provides structural stability, mechanical integrity and enzymatic resilience to collagen-based tissues. This is further enhanced by weak interactions and strong intermolecular crosslinks. Collagen type I is stabilized through the action of four crosslinks: two in the helical region and one more in each telo-peptide, where the action of lysyl oxidase catalyzes the formation of aldehydes from lysine and hydroxylysine residues.[52] The resulting aldehydes react spontaneously with other lysine and hydroxylysine molecules from adjacent chains of the same molecule or from other adjacent molecules. These crosslinks between two different molecules result in head-to-tail bonding along fibrils, known as aldimide bridges.[53] During in vivo biosynthesis, three main crosslinking pathways take place: the lysyl oxidase crosslinking, the sugar-mediated crosslinking and the transglutaminase crosslinking.

3.5.1. Lysyl Oxidase Crosslinking

Beyond the triple helical structure of individual collagen molecules, collagen assemblies receive additional mechanical and chemical stability from crosslinks both between and within component molecules. Intramolecular crosslinks are generated by the action of lysyl oxidase (LO; gene name LOX, EC 1.4.3.13), which engages in PTM of secreted triple helices during fibril formation. While deamidation by LO is prerequisite for the formation of such crosslinks, it merely sets the molecular stage for the spontaneous crosslinking that occurs later. The telo-peptides present at either end of the collagen triple helix are an easy substrate for the enzymes to target, as opposed to the compact triple helix itself. It is here (telo-peptide regions) that LO takes effect, converting selected Lys and Hyl residues to the aldehydes allysine and hydroxyl-allysine, respectively, which can then spontaneously react via aldol condensation during fibrillogenesis (**Figure 3**). Thus, α-chain dimers are produced from intramolecular crosslinks between the telo-peptide sections of two α-chains. These dimers can be observed in SDS-PAGE as β bands.

In contrast, intermolecular crosslinks occur between the telo-peptides of one collagen trimer and the helical region of a quarter-staggered adjacent trimer. One potential trimer bond is the formation of aldimine from an aldehyde residue on one trimer and an ε-amino group of either Lys or Hyl on the other, which yields a bivalent interchain crosslink that is still reactive. Subsequently, multiple condensations with His, Lys, or Hyl residues yield further multivalent crosslinks, which are reducible by sodium borohydride ($NaBH_4$). In most tissues, the number of borohydride-reducible crosslinks decreases with age, most probably because they mature into stable, nonreducible crosslinks.[54] In the case of the Hyl aldehyde pathway, the more mature crosslinks appear to be based on trivalent

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3-hydroxypyridinium residues—Hyl-pyridinoline (3Hyl) and Lys-pyridinoline (2Hyl and 1Lys). The pyridinoline crosslinks withstand proteolytic attack and are released after collagen tissue remodeling. Eventually, they reach the bloodstream and are excreted in the urine, where HPLC or ELISA can be used to quantify this crosslink and consequently assess collagen turnover in the body.[55]

Mature crosslinks are formed later in life and their local concentration depends on the tissue in which they are formed, age, gender, activity, and physical state.[56] In addition to trivalent pyridinolines, another crosslink has been identified in adult cartilage that is formed spontaneously from the initial divalent ketoimines.[57] This arginoline crosslink represents a 3,4-dihydroxy imidazolidine that is formed by condensation of a free arginine with the oxidized ketoimine crosslink. Arginoline content increases with age and is not reducible with sodium borohydride. These findings not only revealed that cartilage collagen II fibrils are more crosslinked than hitherto assumed, but also highlight the importance of crosslinking for load-bearing tissues.

3.5.2. Sugar-Mediated Crosslinking

The discovery of nonenzymatic glycation of hemoglobin molecules in patients with diabetes mellitus triggered investigations into the role of sugars in modifying connective. Specific to collagen, its prolonged exposure to reducing sugars (e.g., ribose and glucose) produces advanced glycation end products (AGEs) that are implicated in aging and diabetic complications.[58] The nonenzymatic glycosylation reaction, which is accelerated in diabetes, is the first step of the Maillard or nonenzymatic browning reaction that occurs in stored food. The glucose–protein adduct rearranges and dehydrates to form brown and fluorescent pigments, which act as crosslinks, resulting in decreased protein solubility and altered mechanical properties. Early studies not only confirmed, but also demonstrated that browning is increased in human collagen over age in a linear fashion and that diabetic patients show accelerated browning, suggesting a correlation between arterial stiffening, decreased joint mobility and severity of microvascular complications in type I diabetes.[59] One should consider that glycation is the major cause of dysfunction of collagenous tissues in old age and the process is significantly accelerated in diabetic patients largely attributed to the higher levels of glucose. Glycation modulates numerous collagen properties, including its biomechanical behavior and supramolecular aggregate assembly. The most damaging effects are due to glucose-mediated intermolecular crosslinks between the triple helices, which decrease flexibility, permeability and turnover. Almost all ECM proteins can react nonenzymatically with a sugar group (frequently glucose) via a free ε-amino group of a Lys or Hyl. This form of glycation is described as Maillard reaction and involves the chemical reaction of a sugar aldehyde or ketone with a free amino group to form Schiff base, which then undergoes rearrangement to form a fairly stable keto–imine (Amadori product). These structures are still reactive and can go on to form AGEs or to degrade into reactive α-dicarbonyl groups, which in turn react with other free amino groups to form crosslinked adducts. These additional (non-LO-facilitated) crosslinks influence the resistance of collagen to degradation and remodeling.[60,61] This appears particularly relevant for collagen-rich tissues, such as dermis, cornea, tendons, ligaments and endomysial sheets of muscles. However, the basement membrane function of macro- and microvasculature (including the blood-filtering glomeruli of the kidney) largely provided by collagen IV is an obvious target for AGE formation and explains the complications of diabetes. Certain molecules, including urea, have been described as AGE breakers and are potential therapeutic targets.

3.5.3. Transglutaminase Crosslinking

Transglutaminase (TGase) mediated collagen to collagen crosslinks were first demonstrated for the highly homologous α1 chains of collagens type V and type XI in cell culture, with an indication that crosslinking occurs in the nontriple helical propeptide domains. TGase activity has also been shown on collagen VIII anchoring fibrils, presumably on crosslinking sites in the NC1 domain of collagen II[62] and on collagen type VII was recently confirmed.[63] In contrast to lysyl oxidase, which facilitates collagen crosslinks, TGases (EC 2.3.2.13) can create them directly. TGases are widely distributed and have been found in microorganisms,[64] across the animal kingdom[65] and recently also in plants.[66] TGases catalyze the formation of an isopeptide bond between the ε amino group of a Lys and the γ carboxamide group of a glutamine. The reaction (transamidation) also produces an ammonia molecule. Depending on the isoenzyme and species the TGase is derived from, the reaction is Ca^{+2} dependent; microbial TGase (mTGase) does not require Ca^{+2}. The resulting isopeptides [ε-(glutamyl)-lysine dipeptides] are very stable and can be isolated from tissue homogenates only after aggressive proteolytic tissue digest. They can also be detected as separate peaks in HPLC, serving as fingerprints of transamidation.[67] Antibodies are also available against the ε-(glutamyl)-lysine crosslink and used as tools to discover transamidated tissue structures. It should be noted that the primary structure of a given protein does not allow prediction of which Lys or Gln might serve as an amine donor or acceptor, respectively. Determination of actual TGase crosslinking sites still requires a good deal of empirical work and direct biochemical analysis. Also, and in contrast to pyridinolines, the identification of an isopeptide bond is not specific for a collagen-to-collagen crosslink; it could also indicate a noncollagen ligand crosslinked with collagen.

There are currently nine TGases known in humans with distinct functions.[68] Knowledge about the exact biochemical activity of TGases was derived from early studies of coagulation protein factor XIII.[69] As stabilizer of fibrin/fibronectin blood clots, FXIIIα has been converted into an industrial product known as fibrin glue (Tisseel). FXIIIα can crosslink fibronectin to collagen, but evidence for the crosslinking of fibrillar collagens is sparse. FXIIIα has been implicated in the crosslinking of the noncollagenous domain of collagen type XVI. The activity of different TGase isoforms can be monitored in tissue cryosections, where the enzyme(s) are still active. Biotinylated peptides, serving as either amine donors/acyl acceptors

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Table 2. Indicative advantages and disadvantages of collagen preparations from various sources.

Source	Advantages	Disadvantages
Tissue extracted collagen	High yield Acid/pepsin extraction removes antigenic p-determinant	Potential of interspecies transmission of disease
Cell synthesized collagen	Can be autologous	Low yield
Recombinantly produced collagen	Low immune response	Low yield Stability issues
Peptide synthesis produced collagen	Would rule out allogeneic/ xenogeneic issues	Low yield Assembly/registration issues

(containing Lys) or amine acceptors/acyl donors (containing Gln), have been successfully employed to localize sites of TGase activity. Conveniently, the offered peptides are irreversibly crosslinked into target structures of the tissue sections or cell cultures and can then be detected with avidin-conjugated probes (enzymes, fluorophores). Thus, TGase activity has been visualized in the cornified envelope of the epidermis[70] and dermis,[71] as well as in connective tissue structures of other organs.[72] As these peptides were designed to be fragments of other ECM molecules, such as fibrillin-1 and osteonectin, these localization studies suggest that TGase 2 may be a modifier of collagen assemblies.

4. Sources of Collagen

To date, numerous collagen preparations are commercially and clinically available; they have been extracted from animal tissues, including human and fish, or from human or land animal cells grown in vitro or have been produced by recombinant expression or direct peptide synthesis. Each of these collagen preparations come with distinct advantages and disadvantages (**Table 2**).

4.1. Extracted Collagen

For biomedical applications, mammalian skin, and tendon tissues (porcine, bovine and ovine in origin) are the primary source of collagen type I, while collagen type II is primarily extracted from bovine, porcine and chicken cartilaginous tissues (**Figure 4**). Type IV collagen is an important component of Episkin (L'Oréal), a reconstituted human epidermis, actively used for the evaluation of the potential toxicity and irritancy of topically applied compounds and as an OECD validated and adopted skin corrosion test.[73] It is worth pointing out that the vast majority of the early work in collagen was carried out using rat-tail tendon collagen due to its high purity and relatively easy extraction process. Waste materials of the fish processing industry (fins, scales and bones) have also been used to extract collagen for the fabrication of biomaterials,[74] but to a smaller extend. Although sponges are the simplest-known multicellular organisms containing collagen, the extraction of collagen from this source is not widely used, though in principle it would be a sustainable source. Pioneering work on the predominantly Mediterranean Sea sponge *Chondrosia reniformis* has shown that collagen from this species is, in contrast to other sources, not soluble in weak acids, but in weak alkaline conditions.[75] The use of sea sponge collagen preparations in tissue engineering is sparse.[76]

Despite the species/tissue origin, collagen is particularly notorious for its large, coherent, covalently crosslinked fibrillar meshwork. To this end, different methods (dilute acidic solutions with or without enzymes, neutral salts, and alkali treatments) are used to isolate and purify different types and amounts of collagen from various tissues, while harsher methods employing heat and acid or alkaline agents (liming) tend to denature collagen to gelatin A or B, respectively, which contain single broken-down triple helices. Dilute acidic solutions effectively disassociate intermolecular aldimine crosslinks (between triple helices), however, they are ineffective against more stable and mature crosslinks (e.g., ketoimine bonds). In this case, proteolytic enzymes (primarily pepsin) are employed, which also increase the yield by up to 10 times.[77–79] Notably,

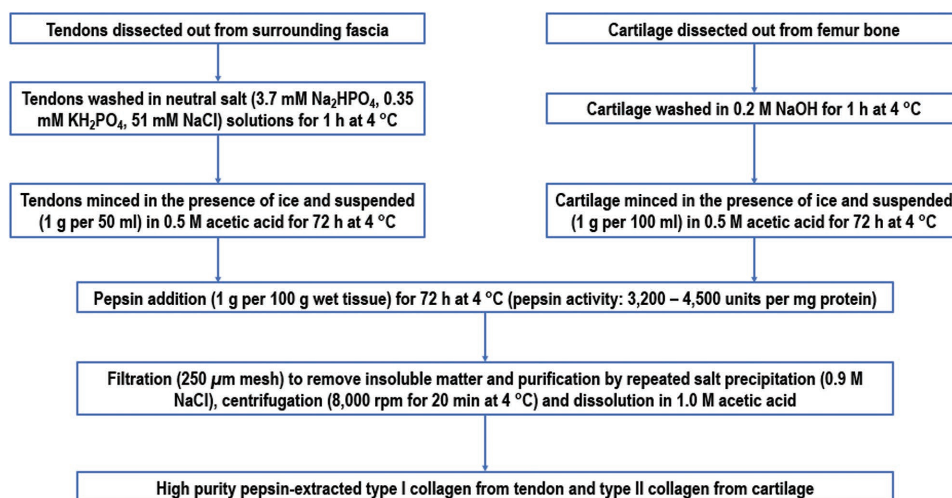

Figure 4. Collagen type I and collagen type II extraction and purification protocol.

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an even partially or locally unfolded triple helix is vulnerable to proteolytic attack, but a tightly folded and intact triple helix is not.[80] The efficacy of enzymatic treatment therefore arises from selective cleavage in the nonhelical N- and C-telo-peptide regions that allows the excision of intact triple helices out of crosslinked fibrillar assemblies.[81] The resulting mono triple helical collagen is named atelocollagen and has been shown to provoke a markedly lower immune response due the removal of the antigenic sequence P-determinant, located at the telopeptide regions.[82]

All advances in extraction and purification procedures aside, collagen is an animal extracted material and therefore raises issues about immunogenicity and interspecies transmission of disease.[83] The triple helical domains of bovine and porcine collagens are highly homologous to human collagen, but immunologically relevant differences lay in the telo-peptide regions may provoke an immune response.[79,84] Although peptic digestion cleaves off the nonhelical ends, the immunogenic potential is not completely eliminated. A much greater concern with xenogeneic biological materials is the transfer of infectious pathogens (e.g., prion disease). These concerns, combined with cultural issues stimulated the investigation into cell-produced collagen, human recombinant collagens and collagen-like synthetic peptides.

4.2. Cell-produced Collagen

As collagens are synthesized by specialized cells, it is plausible to let cells in culture produce these essential ECM molecules and subsequently harvest them either from the media or from the deposited cell-layer. This, however, requires fast- and well-growing cells, with strong biosynthetic activity. Numerous primary and immortalized cells have been used over the years for the production of various collagen types (primarily collagen type I from fibroblasts and collagen type II from chondrocytes) from various species. To enhance collagen synthesis, L-ascorbic acid supplementation is required, given that ascorbate is an essential cofactor in the hydroxylation of collagenous proline and lysine and humans, guinea pigs, primates and other species cannot synthesize ascorbate, due to deficiency in one of the essential enzymes in the liver (gulonolactone oxidase).[85] Low oxygen tension has also been used to increase collagen synthesis up to fivefold in permanently differentiated cells,[86] as hypoxia inducible factor 1 α (HIF 1α) is activated at low oxygen tension cultures and is central regulator of collagen hydroxylation and secretion.[87] Further, low oxygen tension has been shown to upregulate the synthesis of TGF-β1, which is a collagen inducer.[88] It is worth pointing out that this increased collagen synthesis at low oxygen tension (2%) was not mirrored in stem cell cultures, suggesting that activation of HIF-1α alone does not necessarily translate into increased ECM synthesis.[89] Biological factors, in the form of growth factors[90] or gene transfection,[91] have also been recruited as means to increase collagen synthesis. Insect cells[92] and sarcoma cell lines, as is[93] or in combination with recombinant technologies,[94] have also been used as means to produce various collagen types. However, the nonmammalian origin of the former and the cancerous origin of the latter restrict or even prohibit their clinical translation. The yield of collagen from human cells is also very low,[95] limiting further their clinical potential.

4.3. Recombinant Collagen

The use of genetically engineered microorganisms, animals and plants appears to be an alternative option for the production of recombinant human collagens that avoids problems related to batch-to-batch variability, interspecies transmission of disease and xenogeneic immune responses, all of which can be induced by animal extracted collagens.[96] The rational of using microorganisms as means to produce recombinant collagen lays on the fact that evolutionary collagens and collagen-like proteins existed in bacteria before they were present in multicellular organisms.[97] *Saccharomyces cerevisiae*[98] and *Pichia pastoris*[99] yeasts were the first to be investigated, given that as eukaryotes, they are capable of glycosylation. Considering that certain viruses harbor genes encoding prolyl 4-hydroxylase[100] and lysyl hydroxylase,[101] the coexpression of a human collagen type III with mimivirus prolyl and lysyl hydroxylases in *Escherichia coli* has recently been reported.[102] However, the yield of such systems is very low (e.g., 15 mg L^{-1} for yeast,[103] 60 mg L^{-1} for baculovirus,[92] 90 mg L^{-1} in *E. coli*[102]), thus limiting broad commercialization potential. The extraction yield of collagen-like Scl2 protein from *S. pyogenes* was recently improved considerably up to 19 g L^{-1} by combining a stirred bank bioreactor, high cell density and adjusting culture time.[104] Although helical conformation of this collagen-like protein was validated, the enzymatic resistance was not studied. Moreover, this collagen-like Scl2 protein was decorated with heparin, integrin binding or discoidin domain receptors to increase cell adhesion, as Scl2 protein lacks cell binding sites.[105] Another disadvantage of bacterial recombinant collagens is the absence of hydroxyproline. Therefore, bacterial recombinant collagen-like proteins show relatively low denaturation temperature (≈26 °C) and when they are stabilized by electrostatic interaction via multiple interpeptide lysine–aspartate and lysine–glutamate salt bridges,[106] they reach denaturation temperature of 35–39 °C.[107] Tyrosine and cysteine residues have been introduced to induce crosslinks through oxidation.[108] Further, incorporation of Gly–Pro–Ala or Gly–Pro–Hyp peptides has been shown to reduce bacterial invasion of root dentine.[109] This customization was also used to modulate chondrogenesis of human mesenchymal stem cells by incorporating heparin-binding, integrin-binding, and hyaluronic acid-binding peptide sequences into the collagen-like Scl2 protein.[110] Recombinant collagen-like proteins also demonstrated affinity with fibronectin, when it was incorporated into the protein sequence a minimum of six triplets of human collagen type II sequence from residue Gly^{775}–Arg^{792}.[111]

While recombinant collagens have been expressed in a thermally stable triple helical form, they may still differ with respect to proteolytic susceptibility in comparison to native fibrillar collagens.[112] These issues (e.g., low yield, low thermal properties, susceptibility to enzymatic degradation) were tackled with a more complex approach using transgenic animals that secreted procollagen type I trimers into their milk in the mammary glands.[113] Silkworms have also been induced to express a fusion protein of fibroin and collagen.[114] Plants have developed

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an ECM based on carbohydrate polymers and a variety of them possess PTM machinery that includes membrane-bound protein disulfide isomerase/prolyl 4-hydroxylase.[115] To this end, transgenic corn[116] and tobacco[117] plants have successfully been employed to produce human recombinant procollagens. Despite the strides that have been made to date, unicellular organisms do not produce ECM and therefore lack the enzymatic toolbox to post-translationally modify collagen. Thus, in most cases the produced collagens are not stable at peptic digest, suggesting incomplete triple helix formation or thermal instability. Further, the yield is very low for industrial applications, suggesting that a niche area should be identified that would offer opportunities for recombinant technologies to thrive.[118]

4.4. Synthetic Collagens

Trimeric structures of synthetic Gly–X–Y repeats, referred to as collagen-mimicking sequences, collagen-like peptides or collagen-related peptides, are at the forefront of scientific research to address issues associated with animal extracted collagens, cell-produced collagen and recombinantly synthesized collagens.[119] Although advances in synthetic strategies and technologies allow synthesis of long chains, all current synthetic triple helices are below 10 nm in length, thereby falling far short of the classical collagen type I α helix length of 300 nm. Thus, such collagen mimicking synthetic analogues have been used as nanospheres,[120] nanosheets[121] and other microstructures.[122] The problem of registration of alpha chains to form a triple helical domain has been overcome with a sticky-end approach that is related to the strand invasion feature; three short collagen strands (two $[Gly–Pro–Pro]_5$–$[Gly–Pro–Pro]_3$–Cys–Gly and one $[Gly–Hyp–Pro]_3$–Gly–Cys–Gly–$[Gly–Hyp–Pro]_5$) are held in a staggered array by disulfide bonds. The $[Gly–Y–Pro]_3$ segment forms an intramolecular triple helix with a single strand overhang represented by the $[Gly–Hyp–Pro]_5$ stretch (sticky end), allowing annealing of further overhangs of identical trimers to a length of nearly 1 µm in length and 1 nm in diameter. Such systems have the ability to produce collagen-like structures from nano-[123] to microscale.[124] The electrostatic interaction of these oppositely charged amino acids stabilizes the sticky-ended triple helix by forming salt bridges, which have been calculated to increase significantly triple helical stability.[125] The limitation of these materials sets with their production costs. We foresee here a very interesting avenue toward building collagenous, yet synthetic, biomaterials, should their safety and efficacy be demonstrated.

Regardless the source, if collagen is to be part of an implantable medical device, the manufacturing process should include a microbiological safety assessment in conformity with regulatory requirements.[126] For viral inactivation, WHO recommends low pH, solvent and detergent treatments.[127] Sodium hydroxide treatment (1 M, for 1 h at 20 °C) has also shown promise[128]; it should be noted that sodium hydroxide affects collagen stability.[129] Chemical and biochemical contaminants should also be identified and quantified and potentially safety hazards should be documented.[130] With no exception, the final product should entirely comply with ISO 10993,[131] with the in force standard,[132] directives and regulations related to medical devices.[133]

5. Exogenous Collagen Crosslinking

The natural lysyl oxidase-mediated crosslinking of collagen does not occur in vitro and therefore reconstituted collagen assemblies lack sufficient strength and may disintegrate upon handling or collapse under the pressure from surrounding tissues in vivo. Furthermore, the rate of biodegradation has to be customized for the specific application/clinical indication. Thus, it is often necessary to introduce chemical, physical or biological in nature exogenous crosslinks (**Figure 5**) into the molecular structure to tune mechanical properties, to prevent denaturation at 37 °C and to control the degradation rate.[134,135] The fundamental principle of exogenous collagen crosslinking is the formation of covalent bonds between collagen molecules using chemical or natural reagents, which generally link either to the free amine or carboxyl groups of collagen. Although each method (chemical, physical, or biological) provides unique advantages (e.g., tailored to the clinical indication thermal (**Table 3**) and mechanical (**Table 4**) properties), disadvantages (e.g., cytotoxicity at the effective concentration, foreign body response (**Table 5**)) have also been reported, imposing the question "to crosslink or not to crosslink."[135]

5.1. Chemical Methods

The most widely used chemical crosslinking agents are aldehydes (e.g., glutaraldehyde, GTA),[136] isocyanates (e.g., hexamethylene diisocyanate, HMDI),[137] and carbodiimides (e.g., 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC),[138] with variable degree of efficiency. GTA has been shown to extensively stabilize collagen materials because of its self-polymerization capacity that can even crosslink free amines that are relatively far apart.[139] However, degradation products and unreacted GTA, which may remain nonspecifically bound to the matrix, even after exhaustive rinsing with glycine solutions, result in high cytotoxicity.[140,141] Isocyanates also react with amine groups, forming urea linkages and resulting in superior cytocompatibility to GTA, as no potentially toxic side products are formed.[142] In addition, the short half-life of the isocyanates in physiological solutions further enhances their potential in biomedicine.[143] Nonetheless, such potent crosslinking methods are associated with cytotoxicity,[144] calcification[145] and foreign body response,[135,146] even at low concentration, imposing the need for alternative strategies.

Carbohydrates (e.g., ribose[147] and glucose[61]) and plant extracts (e.g., genipin,[148] oleuropein,[149] *myrica rubra*[150]) have also been assessed, but to a smaller extent as the former are associated with pathophysiologies (e.g., diabetes), while the latter may have to face a complex regulatory framework to reach commercialization or clinical translation. The carboxyl groups of aspartic and glutamic acid residues can be used to crosslink collagen through acyl azide (one step reaction)[151] and carbodiimide (two step reaction).[152] EDC/NHS crosslinking involves activation of carboxyl groups, which then spontaneously bond to amine groups of lysine and hydroxylysine residues of collagen. After extensive washing foreign crosslinking molecules are removed, resulting in collagen devices of good cytocompatibility, reduced susceptibility to calcification, but

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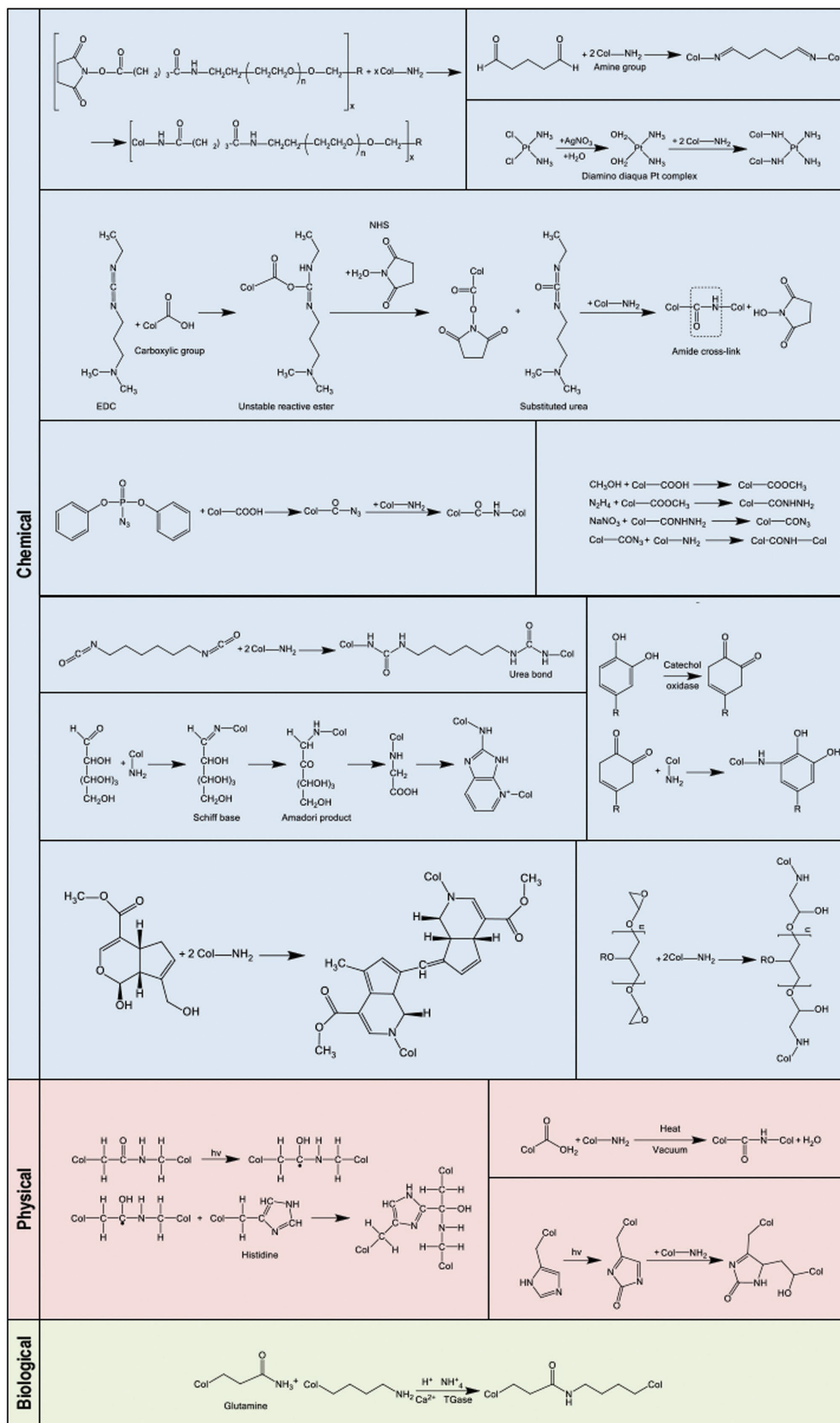

Figure 5. Indicative examples of chemical, physical, and biological in nature exogenous crosslinks that have been utilized over the years to control the properties of collagen-based devices.

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Table 3. Denaturation temperature of collagen-based devices as a function of species, tissue, scaffold conformation, and crosslinking method employed.

Species	Tissue	Scaffold conformation	Crosslinking method	Denaturation temperature [°C]	Ref.
Human	Dermis	Tissue graft	Non-crosslinked	64–67	[223,422]
			Glutaraldehyde	87–88	
			Genipin	81	
Bovine	Tendon	Sponge	Non-crosslinked	79	[423]
			Carbodiimide	80–86	
			Non-crosslinked	62–82	[424]
			Dehydrothermal	54–58	
			Carbodiimide	78–91	
		Extruded fiber	Non-crosslinked	45–47	[134,150,334]
			Dehydrothermal	42–44	
			Ultra-violet irradiation	51	
			Glutaraldehyde	74–76	
			Carbodiimide	56–63	
			Diphenylphosphoryl azide	65	
			Hexamethylene diisocyanate	66–67	
			Genipin	67–68	
			Poly(ethylene glycol) ether tetrasuccinimidyl glutarate	54	
			Transglutaminase	48	
			Myrica rubra	82	
		Film	Non-crosslinked	48	[359]
			Glutaraldehyde	73	
			Genipin	73	
	Dermis	Sponge	Non-crosslinked	49–52	[195,425]
			Glutaraldehyde	48–87	[426]
		Gel	Glutaraldehyde	115–130	[427]
			Carbodiimide	56	[428]
		Electrospun fiber	Non-crosslinked	36–40	[425]
			Carbodiimide	45–60	[429]
Porcine	Tendon	Sponge	Non-crosslinked	53–69	[195,425,430]
			Carbodiimide	86	
		Gel	Non-crosslinked	36–37	[431]
			Carbodiimide	47–49	
		Electrospun fiber	Non-crosslinked	37	[425]
	Dermis	Gel	Non-crosslinked	58	[432]
		Film	Non-crosslinked	36–47	[433]
Fish	Asian sea bass	Sponge	Non-crosslinked	125	[434]
	Asian sea bass	Film	Gamma Irradiation	110–113	[435]
	Jumbo squid	Film	Non-crosslinked	91–108	[436]
	Blue shark	Gel	Non-crosslinked	41	[437]
	Salmon	Film	Ultraviolet irradiation	102	[438]

with reduced mechanical properties and resistance to proteolytic attack.[153]

Recent data advocate the use of branched polyethylene glycol (PEG) polymers,[141,154] but more studies are needed to clearly demonstrate their superiority over conventional chemical approaches.

5.2. Physical Methods

To avoid cytotoxic effects associated with the chemical crosslinkers, physical methods, such as dehydrothermal (DHT)[155–158] and UV irradiation[158–160] and to a lesser extend photo-reactive agents (e.g., rose Bengal[161] and riboflavin[162])

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Table 4. Mechanical properties of collagen-based devices as a function of conformation and crosslinking method employed.

	Type	Crosslinking	Stress	Strain	*E* modulus	Ref.
Native tissue	Skin	Non-crosslinked	1–27.5 MPa	30–180%	4.6–20 MPa	[439]
	Tendon	Non-crosslinked	5–86 MPa	5–22%	1.9–1800 MPa	[440]
	Cornea	Non-crosslinked	120–250 kPa	7–9%	0.1–11.1 MPa	[441]
	Cartilage	Non-crosslinked	0.5–1.0 MPa	7–10%	500–900 kPa	[442]
Biomaterial	Hydrogel	Non-crosslinked	20–27 kPa	25–30%	0.2–100 kPa	[169,177,280,443]
		Glutaraldehyde	10–188 kPa	25–30%	4–100 kPa	[443]
		Carbodiimide	10–172 kPa	20–40%	2–125 kPa	[443,444]
		Transglutaminase	5–10 kPa	30–40%	0.6–1.6 kPa	[168,169]
	Fiber	Non-crosslinked	0.2–4 MPa	12–40%	1–5 MPa	[134,150,334,345,445]
		Glutaraldehyde	8–60 MPa	27–53%	3–47 MPa	
		Carbodiimide	1–4 MPa	23–65%	1–4 MPa	
		Genipin	4–60 MPa	15–43%	2–500 MPa	
		Poly(ethylene glycol) ether tetrasuccinimidyl glutarate	18 MPa	8%	230 MPa	
		Diphenylphosphoryl azide	5 MPa	44%	3 MPa	
		Hexamethylene diisocyanate	17 MPa	45%	4 MPa	
		Transglutaminase	0.1 MPa	61%	0.13 MPa	
		Dehydrothermal	2 MPa	26%	4 MPa	
		Ultraviolet irradiation	3 MPa	21%	6 MPa	
		Myrica rubra	28 MPa	15%	23 MPa	
	Film	Non-crosslinked	1.5–8 MPa	19–50%	1.5–8 MPa	[359,446]
		Glutaraldehyde	8–48 MPa	3–11%	100–1000 MPa	[359,447]
		Carbodiimide	4–20 MPa	30–60%	5–35 MPa	[138,448]
		Genipin	3.5–15 MPa	5–18%	35–130 MPa	[359,446]

Table 5. Advantages and disadvantages of the most widely used exogenous chemical, physical, and biological collagen crosslinking methods.

Crosslinking Method		Advantages	Disadvantages
Chemical	Glutaraldehyde	Very good mechanical properties and resistance to biodegradation	Difficult to control due to self-polymerization capacity Toxicity/inflammation/foreign body response issues
	Hexamethylene diisocyanate	Very good mechanical properties and resistance to biodegradation	Toxicity/inflammation/foreign body response issues
	Carbodiimide	Water-soluble system In general, low toxicity	Low inflammation/foreign body response issues
	Branched polyethylene glycol	Tailored molecular weight and number of functional groups Low toxicity Good mechanical properties and resistance to biodegradation	Very good in vivo response
	Genipin	Good mechanical properties and resistance to biodegradation in general, low toxicity	Low inflammation/foreign body response issues
Physical	Dehydrothermal	Nontoxic	Denaturation issues
	Ultraviolet	Nontoxic	Denaturation issues
Biological	Mammalian transglutaminase	Nontoxic	Expensive Low stability
	Microbial transglutaminase	Nontoxic	Expensive Low stability

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have been assessed. DHT treatment uses high vacuum and temperatures over 100 °C for several hours to promote severe collagen dehydration.[163] Consequently, formation of interchain crosslinks is induced as a result of condensation reactions either by amide formation or esterification between carboxyl and free amino and hydroxyl groups, respectively.[158] UV crosslinking promotes bonds by free radical formation on tyrosine and phenylalanine residues. The crosslinking mechanism is based on the formation of a hydroxyl radical ($OH^•$) from water. The $OH^•$ radical attacks the peptide backbone to produce peptide radicals (−NH−C•−CO−), which can interact to form a crosslink.[159] The efficiency of the reaction depends mainly on the sample preparation, the irradiation dose and time of exposure.[164] It has been reported that UV irradiation of wet collagen fibers causes rapid insolubility[165] and increases their tensile strength.[166] Nonetheless, all physical methods are a lot weaker than the milder chemical method and are often associated with collagen denaturation (especially the DHT treatment), imposing the need for introduction of chemical crosslinks (usually carbodiimide).

5.3. Biological Methods

Tissue-type and microbial TGase have been utilized to stabilize collagen- and gelatin-based materials mimicking the enzymatic in vivo collagen crosslinking pathway. Data to date demonstrate moderate increase in denaturation temperature, mechanical integrity and biological stability, independently of the TGase origin (mammalian or microbial) and the collagen source (mammalian, fish, type I collagen, and type II collagen).[72,167–169]

It is worth pointing out that both physical and biological methods, despite their superior cytocompatibility to chemical approaches, are very weak, often weaker than the mildest chemical approach. Further, the physical methods are associated with collagen denaturation. As such, the quest for the optimal collagen crosslinker continues.

5.4. Collagen Properties Assessment

Over the years, an array of structural, thermal, mechanical, biochemical and biological assays has been developed to analyze/characterize collagen in tissues, cell culture setting, solutions, and 3D scaffold conformations, with variable degree of efficiency, accuracy, and capital infrastructure requirement.

5.4.1. Structural Properties

Collagen molecules self-assemble at nanoscale level to form supramolecular structures (fibrils and then fibers) in the micron range that are visible with various microscopic techniques (**Figure 6**). X-ray diffraction studies have been used in conjugation with TEM analysis to assess the crystalline order of collagenous tissues.[170] Advances in TEM and image processing have allowed the reconstruction of 3D images from serial ultrathin sections to determine collagen assemblies in tissue and their spatial relationship to the cells synthesizing

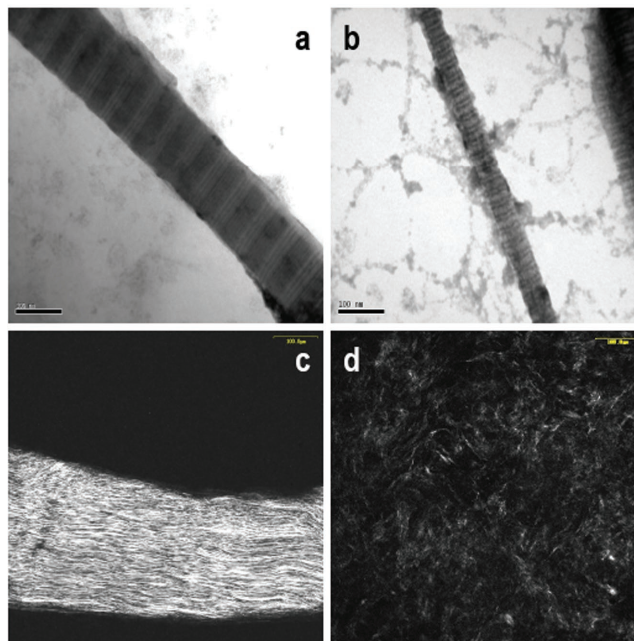

Figure 6. a,b) TEM analyses of rat-tail tendon (a) and self-assembled collagen scaffolds (b) clearly demonstrate the D periodicity/quarter staggered assembly of collagen molecules. c,d) SHG signals of rat-tail tendon (c) are stronger than those of self-assembled collagen hydrogels (d).

them.[171] Scanning electron microscopy is used to study collagen assemblies in tissue context[172] and for imaging collagen-based scaffolds used in the biomaterials field.[173] Time-lapse studies of nanostructures formed by collagen assemblies were conducted,[174] culminating in the real-time monitoring of the kinetics of collagen type I fibrillogenesis on atomically flat mica substrates.[175] Further, time-lapse AFM studies have suggested that collagen fibrils assemble in a two-step process. In a first step, collagen molecules assemble with each other, while during the second step, these molecules rearrange themselves into microfibrils, which are the building blocks of collagen fibers.[176] The in vitro self-assembly process of collagen has also been assessed turbidimetrically and with confocal fluorescence microscopy and is characterized by a lag phase, in which nucleation points form, a growth phase, in which lateral and particularly longitudinal extension of these nuclei into fibers occurs, and a plateau phase, during which no further assembly occurs.[177,178] Raman spectroscopy has also been used for surface imaging of Tyr and Phe rings on assembled collagen fibers.[179]

The observation of cross-striation is a strong indicator for a regular self-assembly, native state, and minimal denaturation. It should be noted, however, that the absence of cross-striation does not signify the absence of collagens, merely the absence of fibrillar collagens. In crystallographic terms, a collagen triple helix can be described as a noncentrosymmetric structure, which after self-assembly into higher-order fibers provides "an ordered nonlinear medium with a cross-sectional path length comparable to near infrared wavelengths."[180] This particular physical feature of collagen fibers allows the observation of optical second-harmonics in multiphoton microscopy.[181]

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Second harmonic generation (SHG) signals have been shown to depend on the order of the structure under observation. For example, skin, tendon, cornea (highly order tissues rich in collagen types I, III, and V) give strong SHG signals, whereas the dermo–epidermal junction (collagen types IV, VII, and XVII) does not. Further, tissues give stronger SHG signals that collagen-based biomaterials.[182]

Various histological stains have been used over the years to assess collagen structures, primarily post implantation. Picrosirius Red staining, for example, of collagenous tissues has been used in conjunction with polarized light microscope to detect fiber quantity and hue.[183] Picrosirius Red consists of elongated dye molecules that readily react with amino acid-rich collagen molecules.[184] Thus, the dye enhances the natural birefringence of collagen by aligning itself in parallel with each collagen molecule.[185] Differences in the birefringence of constituent molecules can be used to identify collagen in a noncollagenous environment and to differentiate individual collagen types, albeit to a certain degree.[186]

5.4.2. Thermal Properties

The C-propeptide is the only area of the procollagen I molecule that can form covalent disulfide bonds to stabilize the procollagen trimer. These bonds lock the C-telo-peptide-mediated registration adjacent to the triple-helical region, where the folding and insertion process of the Gly–X–Y stretches occurs. In addition, chaperones like hsp47 hold the triple helix in shape. In the extracellular space, the removal of the propeptides from procollagen will deprive the triple helix of any covalent bonds that would assist in securing the triple helical conformation of the three α-chains. The tightly coiled triple helix is now held together only by hydrogen bonds and inductive forces created via Hyp residues when facing thermal impact.

The melting temperature 50 (Tm50), at which 50% of a given population of triple helices is molten, can be determined by probing with proteolytic enzymes.[187] Typically, a solution of triple helices is gradually heated and periodically probed at 20 °C with trypsin (targeting the triple-helical domain) and other enzymes that remove propeptides (chymotrypsin, pepsin). Each α-chain contains over seventy consensus sites (www.uniprot.org), which are positioned at the C-terminally of a Lys or an Arg, except when either is bound to a C-terminal Pro.[188] However, these sites are sterically inaccessible, so trypsin can only attack melting regions of the triple helix. During the thermal ramp, α-chains will not loosen progressively, but will melt in cooperative blocks as single structural units. This mode of melting first received attention in microcalorimetric work[18] and was confirmed via studies of the destabilizing effects of single point mutations.[189]

The unfolding of the triple helix shows a steep transition upon heating, whereas refolding occurs in more gradual manner.[190] This is exploited for proteolytic probing at 20 °C after heating. The Tm50 for tryptic measurement has been shown to be 41.5 °C for human collagen I and 39.5 °C for human collagen III,[14] which was validated with circular dichroism spectroscopy.[191] However, it has emerged that the heating rate has major impact on determining Tm50 values; a very slow heating rate (0.004 °C min^{-1}), applied through differential scanning calorimetry (DSC), gave a Tm50 for lung collagen below 36 °C.[192] However, it is worth pointing out that the experiments were carried out in the presence of glycerol, which has been reported not only to inhibit fibril formation of acid and pepsin soluble collagen type I, but also to disassemble already formed fibrils.[193]

In general, DSC is traditionally used to assess the denaturation temperature of medical devices. The high-temperature peak corresponds to the melting of the supramolecular aggregates.[194] Although early studies have assessed the denaturation temperature of various materials in dry state, it has become clear that implants should be incubated overnight in physiological solutions.[195] Given the simplicity of the methods, DSC is extensively used to assess the thermal stability of collagen devices. Data to date clearly illustrate that denaturation temperature is dependent on species, tissues, scaffold conformation/packing density and the extent of crosslinking.[196]

5.4.3. Mechanical Properties

Collagen fibers are responsible for the elastic and viscoelastic properties of the tissues.[197] The primary mechanical strength of collagen results from the self-assembly of collagen molecules into triple helices and collagen fibril which are additionally stabilized by intra- and intermolecular crosslinks.[198] The noncollagenous components are believed to play important roles either through their unique viscoelastic properties (e.g., elastin) or via their interaction with collagen fibers (e.g., glycosaminoglycans and proteoglycans) and allow the tissue to withstand compressive and tensile forces.[199] The length and diameter of the collagen fibers, their spatial distribution, the collagen types present, the content of noncollagenous molecules and the crosslinking content determine the functionality of tissues such as skin, tendon, cornea, blood vessel, cartilage, bone and their mechanical properties.[200]

The deformation mechanism of collagenous structures is similar to those of crystalline polymers that yield and undergo plastic flow and can be divided into four regions: toe or low strain region, heel region, elastic or linear region, and failure (**Figure 7**).[201,202] In general, the slope of the stress–strain curve is increased with strain and this is characteristic of connective tissue.[157,203] The region of low strain corresponds to the gradual removal of a macroscopic crimp in the collagen fibrils and this is visible in the light microscope. The crimp has been shown to act as a buffer or a shock absorber within the tendon, permitting small longitudinal elongation of individual fibrils without damage to the tissue,[198] resulting in its low stiffness.[204] The second stage starts at strains typically beyond 2% strain, after which the effective elastic modulus increases progressively. X-ray studies have demonstrated increase in D-period distance and lateral molecular packing of collagen molecules within fibrils, as a result of the straightening of the collagen kinks. The straightening of the kinks allows fibril elongation and reduction in entropic disorder. The entropic forces increase as the number of kinks decreases, leading to the typical curving upward stress–strain curve.[205,206] The elastic region starts when collagen is stretched beyond the heel region.

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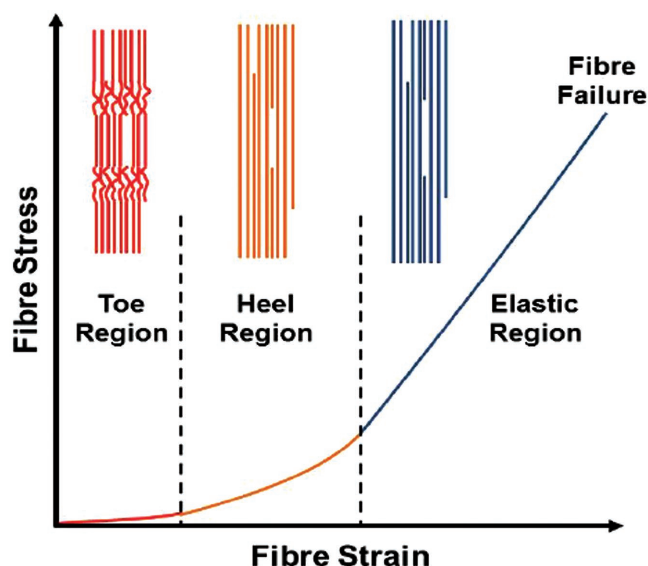

Figure 7. Typical stress–strain curve/deformation mechanism of collagen-based devices depicting the four distinct regions: the toe region, the heel region, the elastic region, and the failure region.

Most kinks are now straightened and no further extension is possible by the entropic mechanism.[202] For larger strains, the exact mechanism by which mechanical energy is translated into molecular and fibrillar deformation is still unclear; most probably, large strain rates indicate stretching of the triple helixes and fiber slippage, resulting in lengthening of the gap region with respect to the length of the overlap region, implying a side-by-side gliding of collagen fibrils.[202,207] During loading at large strains, collagen hierarchical structure is extensively deformed and fibrils can split into individual microfibrils. The collagen network ruptures when several microfibrils break up, a process termed defibrillation.[206,208,209]

5.4.4. Biochemical and Biological Properties

Various assays are available to assess the purity, concentration, and crosslinking density of collagen-based materials.[210] Collagen extracted from different tissue sources and cell layers (**Figure 8**) can be characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weight, charge, size and shape.[211] Protein bands are subsequently visualized using Coomassie Brilliant Blue or silver staining (over 40-fold more sensitive than Coomassie Brilliant Blue) and quantified by densitometry.[78,212] Delayed and reduced electrophoresis can be used to separate α1(III) chains from α1(I) chains.[213] To determine collagen content, hydroxyproline assay is customarily used,[214] although metabolic labeling with radioactive amino acids,[215] high-performance liquid chromatography[216] and colorimetric assays have been proposed.[217] A rather simplified colorimetric assay has also been introduced (Sircol Collagen Assay, Biocolor Ltd., Northern Ireland) for the quantification of collagen in cell and tissue culture.[218] However, the binding capacity of Sirius Red with the side chain of basic amino acids overestimates collagen content. To this end, a pepsin digestion step followed by column ultrafiltration purification step has been recommended to increase the accuracy of the assay.[219] Ninhydrin assay is utilized to quantify the amount of free amino acids. Ninhydrin reacts with the primary free amino groups of the protein and a color change, from yellow to purple (Ruhemann's purple), occurs.[220] 2,4,6-Trinitrobenzene sulfonic acid (TNBSA) assay is also used

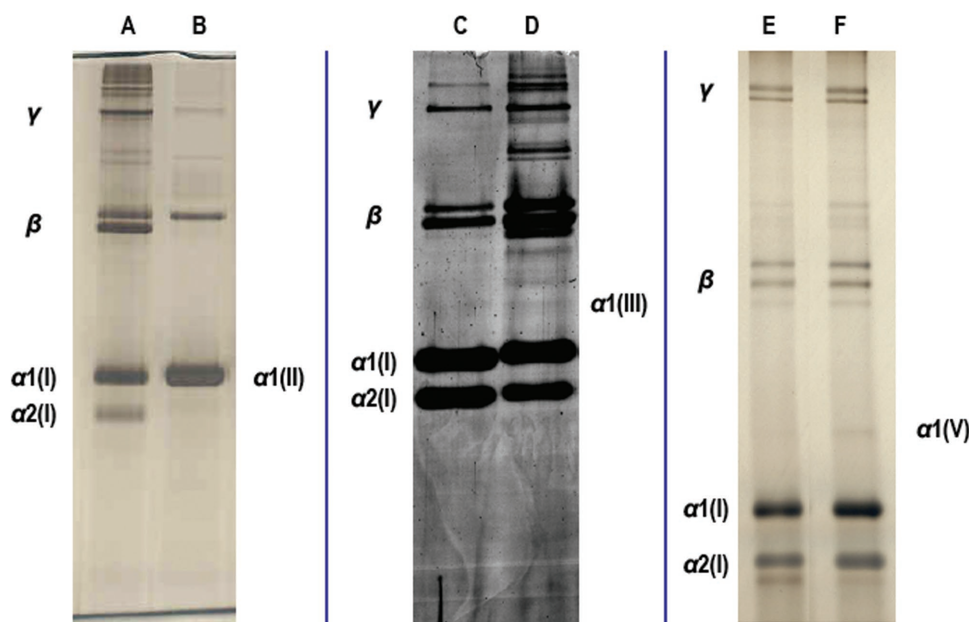

Figure 8. SDS-PAGE of collagen preparations from different tissues and cell layers. A) Porcine achilles tendon collagen. B) Porcine articular cartilage collagen. C) Bovine achilles tendon collagen. D) Porcine skin collagen. E) Dermal fibroblast deposited collagen. F) Mesenchymal stem cell deposited collagen.

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as means to quantify free amino groups. The concentration of N-trinitrophenyl protein derivatives is measured by molecular absorption spectroscopy at 345 nm.[221] In vitro enzymatic degradation of collagen-based devices by matrix metalloproteinases, usually MMP-1,[222,223] allows investigation of the stability of the devices.[224] However, MMP-1 preferentially cleaves collagen type III, as opposed to MMP-8, which is the predominant collagenase present in normal wound healing and degrades collagen type I more efficiently than MMP-1.[225] MMP-1, 2, 8, 13, and 14 are capable of hydrolyzing collagen types I, II, and III, while MMP-3 and 9 are unable to degrade tropocollagen.[226]

6. Collagen Scaffolds

Collagen-based devices, in various physical forms, are extensively used in biomedicine (**Figure 9**). Current and emerging scaffold fabrication technologies aspire to recapitulate the complex native tissue structural hierarchy and mechanical integrity.[227] Obviously, decellularized tissues achieve maximum structural biomimicry, but suffer from limited availability (autografts) and potential immune response (allografts and xenografts).[228] Mechanical loading has been used as a means to develop aligned and densified collagen gels, but further optimization is needed to mimic the complexity of native tissues.[228,229] Electrospinning has enabled the development of 3D tissue equivalents, however, controlling spatially fiber distribution is still challenging, dense constructs limit cell infiltration and the solvents used induce collagen denaturation.[182,230] This section provides a short overview on recent advancements in tissue grafts, hydrogels, sponges, fibers, films, hollow spheres, and tissue-engineered living substitutes.

6.1. Tissue Grafts

Autologous, allogeneic, or xenogeneic tissue grafts are well-established implantable devices due to their similarity with the tissue to be replaced and their complex molecular and biological content that allows cell attachment and promotes spatial cell organization.[231] Given the limited availability of autografts, allogeneic and xenogeneic skin,[223] small intestine submucosa,[232] bladder,[233,234] pericardium,[136] skeletal muscle,[235] heart valve,[236] tendon,[237] and ligament[238] grafts are extensively used in clinic and are often considered as the gold standard.

A typical manufacturing process of tissue grafts consists of depilation (for skin), physical isolation of surrounding tissues, decellularization, crosslinking, disinfection, freeze-drying, and sterilization. All processing steps should maintain as much as possible of the original composition, structure, mechanical integrity, and bioactivity of the tissue.[239] Decellularization is an inherent part of the process aiming to remove cells, DNA, cellular debris, and any other molecules that can act as an immunogen or incite an inflammatory response when implanted.[240] Several decellularization methods are available combining chemical, biological and physical treatments with variable degree of efficiency with respect to ECM disruption.[241]

Although chemical crosslinking methods are extensively used to control mechanical stability and degradation rate, an optimal method has still to be identified.[135] Data to date demonstrate that chemical crosslinking methods at low concentration alter wound healing, while at high concentration are associated with cytotoxicity, proinflammatory macrophage response, inhibition of macrophage polarization, reduced cell infiltration, and delayed wound healing, often resulting in peri-implantation fibrosis.[135,242] Lyophilization is frequently used to increase product longevity and to avoid matrix disruption during sterilization.[239] With respect to sterilization, chemical approaches (e.g., ethylene oxide[243]) are associated with cytotoxicity, while physical methods (e.g., gamma irradiation[244] and e-beam irradiation[245]) are associated with decreased mechanical properties, subject to the device's physical characteristics, suggesting that the sterilization method to be used is device-dependent.[246]

Each clinical application requires different material properties and this has encouraged companies to produce several different ECM materials (**Table 6**). For example, small intestine

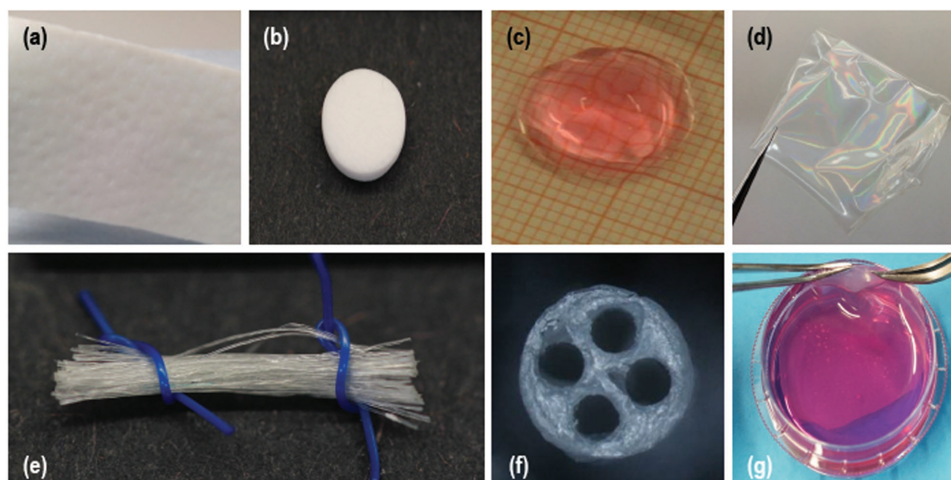

Figure 9. Indicative examples of collagen-based devices utilized in biomedicine. a) Acellular porcine dermal tissue graft. b) Freeze-dried collagen type I sponge. c) Collagen type II hydrogel loaded with cells. d) Transparent collagen type I film. e) A bundle of extruded collagen type I fibers. f) Multichannel collagen type I neural conduit. g) ECM-rich living tissue substitutes produced in vitro using primary human skin fibroblasts under macromolecular crowding conditions.

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Table 6. Indicative examples of clinically available tissues grafts, for various clinical indications, provided along with their properties.

Product and company name	Product details	Clinical indication	Properties
AlloDerm, LifeCell	Acellular non-crosslinked human dermis Sterilization: electron beam irradiation	Soft tissue repair (e.g., hernia and breast reconstruction)	Degradation temperature: 64–67 °C Max. tensile strength: 19–21 MPa Ball burst strength: 800–1200 N cm^{-1} Degradation profile: >12 months
Allomax, Bard-Davol	Acellular non-crosslinked human dermis Sterilization: gamma irradiation	Soft tissue repair (e.g., hernia, thoracic wall and breast reconstruction)	Degradation temperature: 53–55 °C Max. tensile strength: 13–15 MPa Ball burst strength: 230–350 N cm^{-1} Degradation profile: >6 months
Collamend, Bard-Davol	Acellular porcine dermis crosslinked with EDC Sterilization: ethylene oxide	Soft tissue repair (e.g., hernia)	Degradation temperature: 62–67 °C Max. tensile strength: 8–14 MPa Ball burst strength: 64–120 N cm^{-1} Degradation profile: >12 months
FlexHD, Ethicon	Acellular non-crosslinked human dermis Sterilization: ethanol	Soft tissue repair (e.g., hernia)	Degradation temperature: 62–64 °C Max. tensile strength: 12–17 MPa Ball burst strength: 730–1130 N cm^{-1} Degradation profile: >12 months
Permacol, Medtronic	Acellular porcine dermis crosslinked with HMDI Sterilization: gamma irradiation	Soft tissue repair (e.g., hernia)	Degradation temperature: 60–61 °C Max. tensile strength: 7–10 MPa Ball burst strength: 55–75 N cm^{-1} Degradation profile: >24 months
PeriGuard, Synovis Surgical	Acellular bovine pericardium crosslinked with GTA Sterilization: ethanol and propylene oxide	Soft tissue repair (e.g., thoracic wall, hernia)	Degradation temperature: 83–85 °C Max. tensile strength: 20–23 MPa Ball burst strength: 85–115 N cm^{-1} Degradation profile: >24 months
Strattice, LifeCell	Acellular non-crosslinked porcine dermis Sterilization: electron beam irradiation	Soft tissue repair (e.g., hernia)	Degradation temperature: 60–62 °C Max. tensile strength: 9–11 MPa Ball burst strength: 230–320 N cm^{-1} Degradation profile: >6 months
SurgiMend, TEI Biosciences	Acellular non-crosslinked bovine dermis Sterilization: ethylene oxide	Soft tissue repair (e.g., general and plastic reconstruction)	Degradation temperature: 57–58 °C Max. tensile strength: 26–30 MPa Ball burst strength: 415–445 N cm^{-1} Degradation profile: >6 months
Surgisis, Cook Medical	Acellular non-crosslinked porcine small intestine submucosa Sterilization: ethylene oxide	Soft tissue repair (e.g., pelvic organ prolapse, hernia)	Degradation temperature: 61–62 °C Max. tensile strength: 2–3 MPa Ball burst strength: 195–205 N cm^{-1} Degradation profile: <6 months
Veritas, Synovis Surgical	Acellular non-crosslinked bovine pericardium Sterilization: irradiation	Soft tissue repair (e.g., hernia)	Degradation temperature: 44–46 °C Max. tensile strength: 7–11 MPa Ball burst strength: 120–130 N cm^{-1} Degradation profile: >6 months
XenMatrix, Bard-Davol	Acellular non-crosslinked porcine dermis Sterilization: electron beam irradiation	Soft tissue repair (e.g., hernia)	Degradation temperature: 53–55 °C Max. tensile strength: 11–12 MPa Ball burst strength: 330–410 N cm^{-1} Degradation profile: <6 months

submucosa and bladder have been used for applications that require rapid cell infiltration, matrix degradation and remodeling that lack high mechanical performance, including certain types of hernia,[146] rotator cuff tendon repair,[247] bladder surgery,[248] pelvic organ prolapse repair,[249] cardiovascular surgery,[250] or general wound healing (ulcer, burns, and skin substitute).[233,251] On the other hand, skin-derived materials are used for applications that require higher mechanical performance and enzymatic resistance, such as ventral and abdominal hernia repair[146,252] and infected wounds.[253] Recent efforts are directed toward functionalization of tissue grafts to enhance further their biological activity.[254] Despite the significant strides that have been achieved in the field, immune response and delayed remodeling[255] have stimulated research into scaffold-based approaches.

6.2. Self-Assembled Hydrogels

Hydrogels are water-swollen structures that resemble the properties of soft tissues more closely than any other type of polymeric biomaterial.[256] Collagen has the ability to

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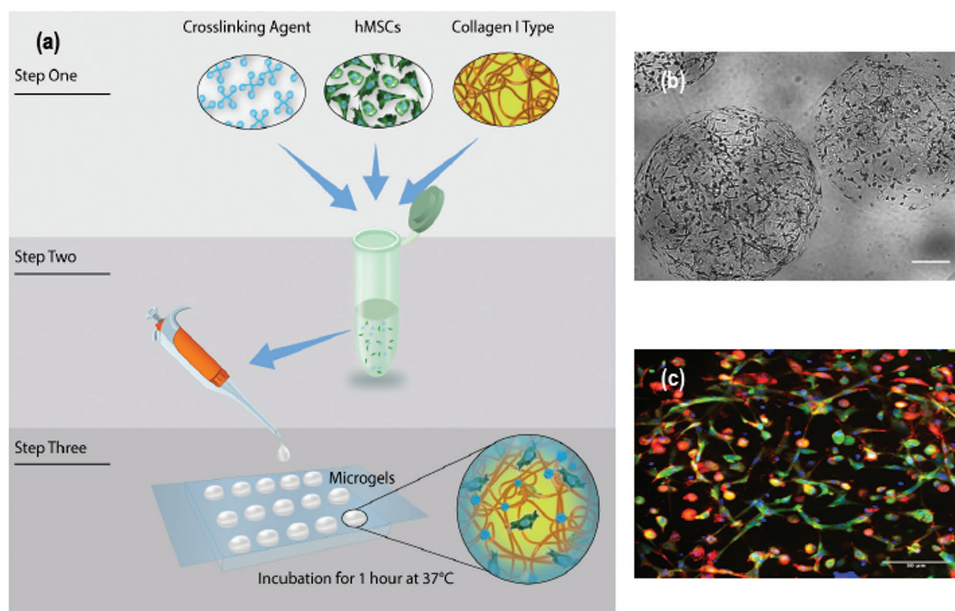

Figure 10. a) Collagen type I microgel fabrication process. b) Bright-field microscopy image of collagen type I microgels loaded with human mesenchymal stem cells after 48 h in culture (c).

polymerize in vitro into a fibrillar hydrogel at physiological pH, ionic strength and temperature, following an entropy-driven process.[257] The intertwined fibrillar substructure is held together by electrostatic and hydrophobic bonds[258] and entraps huge amounts of fluids, permitting that way the exchange of ions and metabolites with surrounding tissues.[259] The flowable nature of collagen hydrogels is primarily attributed to this high liquid phase and along with their fast assembly time (<10 min) at physiological pH and temperature allow them act as injectable systems and ideal carriers for cells and therapeutic/bioactive molecules.[260] Crosslinking offers control over the liquid content and influences the mechanical properties and the degradation profile of the resultant hydrogels.[261] An alternative strategy to improve the mechanical properties of the hydrogels is based on confined and unconfined plastic compression.[262] Advances in engineering have also enabled the development of spherical collagen type I[263] and collagen type II[263] microgels (**Figure 10**).

These unique properties of collagen hydrogels have made them the scaffold of choice for numerous clinical indications. In soft tissue repair, for example, collagen type I hydrogels seeded with fibroblasts exhibited a compact structure similar to that of dermis.[264] Skeletal muscle derived stem cells loaded into a collagen type I hydrogel increased the expression of cardiac genes and similar contractile forces and intracellular calcium ion transients were observed as that of native cardiac cells.[265] When collagen type I hydrogels were subjected to mechanical tension, embryonic stem cells were differentiated to cardiomyocytes,[266] while cardiomyocytes loaded collagen type I hydrogels resulted in formation of cardiac muscle bundles, resembling adult cardiac tissue.[267] In the neural space, collagen type I hydrogels, alone or in combination with growth factors and polypeptides, have been shown to promote polarity of neurons[268] and to align and improve neural cell adhesion, survival and growth.[269] Glyco-mimetic functionalized collagen type I hydrogels have been shown to encourage sensory and motor neuron outgrowth and enhance Schwann cell proliferation and extension.[270] Growth factor loaded collagen type I hydrogels have also shown potential in central nervous system applications.[271] In the eye space, collagen type I hydrogels (noncompressed and compressed) have been used as substrates to grow various ocular-specific cell populations.[272] In tendon repair and regeneration, collagen type I hydrogels have been used either as a means to expand tenocytes in vitro[273] or to improve cell retention of another device with adequate mechanical properties.[274] Collagen type I[275,276] and collagen type II[277] hydrogels have been used extensively for osteochondral and cartilage defect repair, respectively. Collagen II is a typical cartilage collagen. It therefore makes sense that collagen type II hydrogels, as opposed to collagen type I hydrogels, maintain chondrocyte phenotype[278] and drive mesenchymal stem cell differentiation toward chondrogenic lineage.[279]

Numerous preclinical data are also available advocating the use of collagen hydrogels for numerous clinical targets. In skin, for example, collagen type I hydrogels have displayed good integration and they were colonized by host cells within 15 d.[280,281] In the neural field, collagen hydrogels loaded with growth factors have shown promise in rat spinal cord injury models[271,282] and in rat sciatic nerve models.[283] In a rabbit corneal keratitis model, a collagen type I hydrogel loaded with a drug inhibited bacterial growth and maintained corneal clarity.[284] In a rabbit Achilles tendon gap model (collagen hydrogels are not suitable for large defects due to low mechanical integrity) collagen type I hydrogels were used as carriers of mesenchymal stem cells, resulting in improved structural and functional outcomes.[285] In a cartilage sheep model, collagen type I hydrogels containing autologous mesenchymal stem cells that had been differentiated into chondrocytes resulted in cartilage regeneration,

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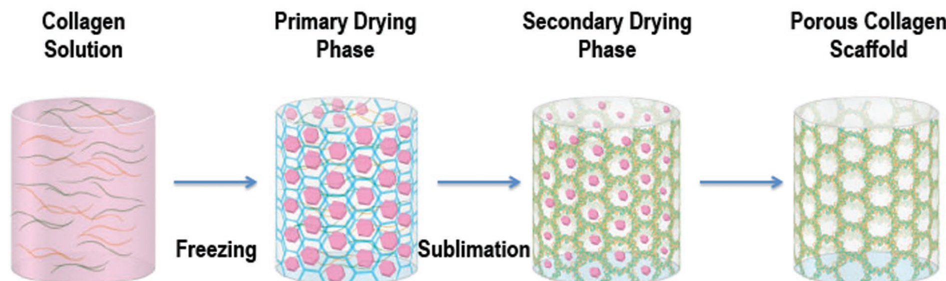

Figure 11. Porous collagen scaffolds are fabricated using freeze-drying. By adjusting the freezing rate, the size and the porosity of the sponge can be effectively controlled. Specifically, primary freezing at high temperature increases the pore size through the formation of large ice crystals, whereas freezing at low temperature decreases the pore size through the formation of small ice crystals.

although it us worth pointing out that areas of incomplete integration and cyst formation were observed.[275]

Significant have also been the strides with collagen hydrogels in clinical setting. Apligraf is a living bioengineered system made out of a collagen type I hydrogel and allogeneic fibroblasts and keratinocytes. This system has been used successfully in clinic for skin replacement, burn wounds and diabetic foot ulcers.[264,286] However, drawbacks such as extensive shrinkage, poor porosity and poor persistence of fibroblasts within the hydrogel have been reported.[287] A collagen type I hydrogel loaded with bone marrow mesenchymal stem cells has also been used successfully in myocardium.[288] Numerous studies have also demonstrated the potential of collagen type I hydrogels loaded with chondrocytes or mesenchymal stem cells for cartilage repair.[289] The potential of human recombinant type III collagen has also been demonstrated in clinical setting for corneal repair.[290]

6.3. Freeze-Dried Sponges

Freeze-drying (also known as ice crystal templating or lyophilization or ice-segregation-induced self-assembly) is a dehydration process that can be used for the construction of highly porous implantable devices (**Figure 11**) for a diverse range of clinical indications (**Table 7**). Upon freezing, collagen is entrapped within the developing ice crystals, which have formed into hexagonal structures. The porosity of the collagen sponge can be controlled by the freeze-drying rate and after sublimation of the ice crystals in the course of the drying phase.[291] For optimal bioactivity, the pores should be large enough to permit the migration of cells and diffusion of nutrients and small enough to promote cell attachment.[292] However, too small pores should be avoided, as they restrict cell attachment and differentiation potential.[293–295]

Numerous molecules and cell populations have been used to date to enhance further the bioactivity of collagen sponges with promising results in both in vitro and in vivo settings. A collagen-glycosaminoglycan scaffold has been shown to enhance in vitro osteogenesis in human osteoblast culture[296] and to induce osteogenic and chondrogenic differentiation of adult rat mesenchymal stem cells.[297] Fibrin networks incorporated into a collagen sponge improved osteoblast attachment, proliferation and differentiation.[298] A collagen/hydroxyapatite/chondroitin sulfate sponge has been shown to differentiate stem cells toward chondrogenic lineage and to simulate cartilage-like ECM synthesis.[299] In preclinical models, collagen-glycosaminoglycan and collagen-calcium phosphate scaffolds have been shown to repair rat calvarial defects as effectively as autologous bone materials and more effectively than scaffolds loaded with mesenchymal stem cells.[300] Collagen/recombinant human bone morphogenetic protein 2 scaffolds enhanced osteoclastogenesis, osteoblastogenesis and osteoclast activation and increased bone volume and the expression of bone resorption and formation markers, without adverse healing events (e.g., swelling, excessive bone formation, and seroma formation) in a rat calvarial defect model.[301] A collagen-hydroxyapatite sponge loaded with recombinant human bone morphogenetic protein 2 has been shown to increase healing in critical size rat calvarial defect within 8 week post-implantation, without provoking bone anomalies or adjacent bone resorption.[302] A collagen/rosuvastatin sponge has been shown to enhance bone formation in critical size proximal tibial cortical bone of New Zealand White rabbits, as evidenced by increased in BMP-2 mRNA levels, higher bone volume, increased bone mineral density and new bone formation.[303] Collagen sponges, alone[304] or in combination with hyaluronic acid/hydroxyapatite/beta-tricalcium phosphate[305] or with osteogenic protein,[306] have been shown regenerative capacity in osteochondral defects of rabbits and minipigs, as evidenced by increased gene expression of cartilage molecules (e.g., collagen type II, aggrecan, and SOX9) and improved biomechanics. When skin-derived precursors loaded on a collagen sponge and implanted to the wound areas of diabetic mice, accelerated wound healing and enhanced local capillary regeneration was observed by day 14,[307] while collagen sponges loaded with adult bone marrow mesenchymal stem cells showed a high density of vascularization in immunodeficient mice.[308] Collagen/gelatin sponges loaded with basic fibroblast growth factor[309] or concentrated platelet lysate[310] have been shown to regenerate full-thickness defects on the backs of normal mice, on the palatal mucosa of dogs and on pressure-induced decubitus ulcer of genetically diabetic mice, as evidenced by neo-epithelium length and total area of newly formed capillaries assessment and accelerated wound healing. Collagen sponges containing latent TGF-β binding protein 4 stimulated elastic fiber growth, when implanted between the dermis and cutaneous muscle on the backs of athymic nude mice.[311]

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Table 7. Indicative examples of FDA-approved collagen sponges for various clinical indications.

Product and company name	Characteristics	Clinical Indication
Avitene UltraFoam, Bard-Davol	Purified bovine corium collagen sponge Sterilization: dry heat	Hemostasis
CollaGraft, Zimmer	Bovine collagen sponge containing hydroxyapatite/tricalcium phosphate granules Sterilization: N/A	Bone
COLLARX, Innocoll	Bovine or equine collagen sponge COLLARX with gentamicin (INL-002) or bupivacaine (INL-001) Sterilization: N/A	Wound healing
CopiOs, Zimmer Biomet	Bovine collagen sponge-containing dibasic calcium phosphate Sterilization: N/A	Bone
GENTA-COLL resorb, Resorba	Equine collagen sponge containing gentamicin Sterilization: N/A	Soft tissue wounds; abscess caverns; joint empyema; spongioplasty; osteitis, osteomyelitis; implant associated infections; diabetic foot; extirpation of the rectum; pilonidal sinus; ano-rectal injuries; sternotomy; heart pacemaker replacement
INFUSE, Medtronic	Collagen sponge containing recombinant human bone morphogenic protein 2 Species: N/A Sterilization: nanofiltration	Bone
Integra Mozaik, Integra	Bovine collagen (20%) and tricalcium phosphate (80%) sponge Sterilization: irradiation	Bone
KOLLAGEN resorb	Equine collagen sponge Sterilization: N/A	General surgery; gynecology; thoracic and cardiovascular surgery; orthopedic and trauma surgery; maxillary surgery and ENT, hemostasis
Ologen, Aeon Astron	Porcine collagen (≈90%) and glycosaminoglycans (≈10%) sponge Sterilization: N/A	Glaucoma surgeries; glaucoma drainage; strabismus; pterygium; revision surgeries
OssiMend, Collagen Matrix	Bovine collagen sponge containing 55% bone mineral Sterilization: N/A	Bone
Zimmer Collagen Plug, Tape, Patch, Zimmer Biomet	Bovine collagen sponge Sterilization: gamma irradiation	Denture sores; oral ulcers (noninfected nor viral); periodontal surgical wounds; suture sites; burns; extraction sites; surgical wounds; traumatic wounds

Numerous data have also advocated the use of collagen sponges, with or without functional molecules and/or cells, in clinical (human) setting. Collagen sponges have been shown to induce a substantial increase in the connective tissue thickness of palatal.[312] Collagen sponges have been shown to be more effective than autologous tissues in cranial neurosurgery.[313] Collagen sponges loaded with recombinant human bone morphogenetic protein 12 have been used successfully in rotator cuff surgery.[314] Gentamicin, Cefaclor or Ranalexin loaded collagen sponges have been used successfully in diabetic foot,[315] cochlear,[316] sternal,[317] abdominal,[318] thoracic[319] and cardiac[320] infections. A collagen/gelatin/basic fibroblast growth factor has shown promise in chronic skin ulcers treatment.[321] Recombinant human bone morphogenetic protein-2 combined with a collagen sponge resulted in a relatively shorter fusion time, but increased risk of posterior cervical wound complications may rise in posterolateral lumbar spine fusion.[322] A collagen sponge with autologous chondrocytes has shown good short-term clinical and radiological results in large focal chondral and osteochondral defects.[323] A collagen sponge loaded with autologous mesenchymal stem cells has also been used successfully in intervertebral disc regeneration, as evidenced by radiograph, computed tomography and magnetic resonance imaging analysis.[324] CD34+ cell delivered with a collagen sponge containing recombinant human bone morphogenetic protein 2 achieved mature bone regeneration and increased bone density and mean trabecular bone area.[325]

Given that traditional freeze-drying processes produce scaffolds with random architecture, advances in freeze-drying technologies offer control over the ice crystal formation and segregation, enabling the development of highly ordered collagen sponges that closely imitate native supramolecular assemblies.[326] Such scaffolds have induced in vitro tenocyte[327] and neurite[328] elongation and formation of homogenous cartilage-like tissue.[329] Preliminary in vivo data are also promising.[295,307]

6.4. Self-Assembled Fibers

Although the benefits of electrospinning are well known by now,[330] unfortunately, electrospinning of collagen still remains a challenge, as the current process leads to irreversible denaturation.[182,230] For this reason, extruded collagen fibers and isoelectric focusing produced fibers are discussed here. Collagen fibers, with structural and mechanical properties similar to native tissues, have been produced through the extrusion of a collagen solution in a series of phosphate buffers maintained

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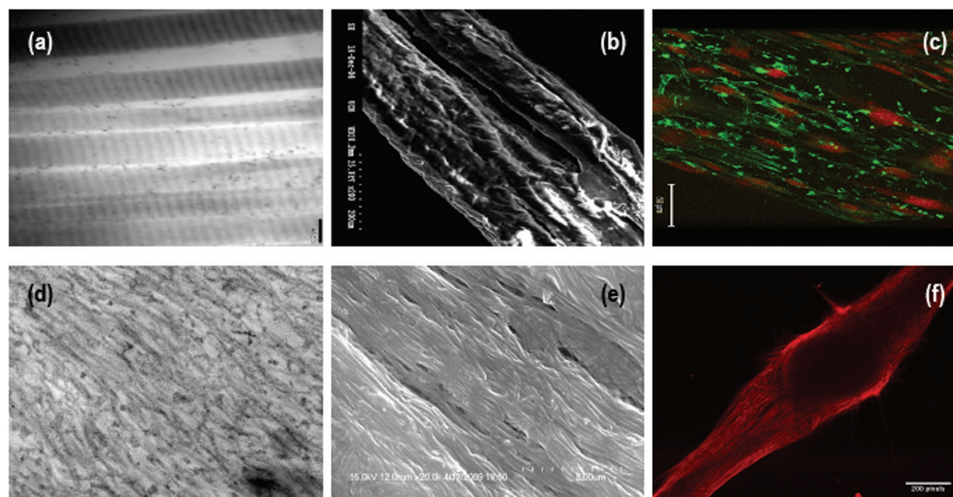

Figure 12. a,d) TEM analyses of extruded collagen fibers (a) and isoelectric focusing produced fibers (d) illustrate bidirectional subfibrillar architecture and the characteristic D-periodicity of collagen. b,e) SEM analyses of extruded collagen fibers (b) and isoelectric focusing produced fibers (e) illustrate that the bidirectional subfibrillar architecture induces a bidirectional surface topography. c,f) This bidirectional surface topography induces bidirectional human tenocyte growth on extruded collagen fibers (c) and bidirectional rat embryonic dorsal root ganglion explants growth on isoelectric collagen fibers (f).

at 37 °C.[157,207,209,331] Collagen extraction method, collagen concentration, extrusion tube diameter, composition of the phosphate buffers and crosslinking method offer opportunities to tailor the mechanical properties of the fibers to the clinical target of interest.[78,332] Undulation and crevices running parallel to the longitudinal fiber axis (**Figure 12**) have been shown to enhance cell attachment and to promote bidirectional cell growth[333,334] and neotissue formation.[335–337] To enhance further the biological and biophysical properties of these fibers, functionalization strategies with decorin[207] and resilin[338] have been proposed. Such materials have also shown great in vivo outcomes in various preclinical models. For example, minor inflammatory reaction and biological degradation within 6 week postimplantation have been reported in a mouse subcutaneous model.[339] In an ovine tendon model, although the collagen fibers were nicely integrated and the tissue was regenerated, the rate of resorption was quite low due to high levels of crosslinking.[337] In rabbit models, carbodiimide and dehydrothermal/carbodiimide crosslinked fibers induced neotendon tissue with mechanical properties and structural characteristics similar to normal tendon tissue within 10–52 week postimplantation, while glutaraldehyde crosslinked fibers formed capsule and inflammation.[335,340] In anterior cruciate ligament rabbit[341] and dog[336] models, these fibers achieved complete regeneration within 12 week postimplantation.

An alternative strategy to prepare anisotropic collagen fibers is based on the principles of isoelectric focusing, which induces the collagen monomers to migrate toward and focusing at their isoelectric focusing point, where the overall charge is neutral.[342] The produced fibers have structural and mechanical properties similar to native tissues.[343–345] These aligned collagen fibers have been shown to provide topographical cues for in vitro bidirectional axonal guidance (Figure 12), even in the presence of myelin-associated glycoprotein that is known to inhibit neurite guidance.[343] These anisotropic substrates have also been shown to induce bidirectional growth of tendon-derived fibroblasts and bone marrow stromal cells[346] and to stimulate tenogenic differentiation of bone marrow stem cells.[347] In a rabbit patellar tendon model, these fibers were gradually degraded over 8 month period.[348] Further, aligned collagen fibers have been demonstrated to improve bone[349] and vascular[350] differentiation.

6.5. Collagen Films and Tubes

Isotropic collagen films, produced through evaporation, have been used extensively in biomedicine for cornea repair due to their transparent nature and the low mechanical requirement of the tissue.[351] Indicative in vitro data have demonstrated that collagen films with thickness of 2 µm, comparative to Bruch's membrane, supported growth of ARPE-19 cells (a retinal pigment epithelia cell line), maintained physiological cell morphology and the cells developed epithelium characteristics.[352] Collagen films, alone or in combination with gelatin or hyaluronic acid and crosslinked with carbodiimide, exhibited similar diffusion and mechanical properties to human cornea and supported growth of human corneal epithelial cells.[353] Collagen films functionalized with tobramycin and crosslinked with carbodiimide, exhibited prolonged antibiotic release and human corneal epithelial cell adherence and growth.[354] Collagen films, having lamellae-like substructure, have been shown to support growth of stromal fibroblasts.[355] In the wound healing area, collagen films, alone[356] or functionalized with Indian Lilac tree extract,[357] have been shown to maintain growth of rat epidermal cells, to withheld collagenase degradation and to reduce nitric oxide synthesis in RAW 264.7 culture. In the lung space, collagen films supported pulmonary stem cell attachment and growth,[358] while collagen films functionalized with Ficoll and crosslinked with genipin supported attachment and growth of WI38 fibroblasts.[359] Given that collagen films wrapped in a single channel tubular conformation have resulted in axonal

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dispersion, multichannel EDC/NHS crosslinked conduits have been used with in vitro data demonstrating high denaturation temperature, resistance to enzymatic degradation, maintenance of structural conformity for up to 30 d in saline solution, superior to single-channel conduits mechanical properties and unaffected neurite outgrowth of dorsal root ganglia explants.[360]

In a rabbit model, collagen films functionalized with tobramycin and crosslinked with carbodiimide facilitated wound healing completion within 15 d postimplantation and by month 3 neovascularization was observed.[354] Again in a rabbit model, collagen membranes functionalized with citric acid and crosslinked with carbodiimide displayed suitable tensile properties and 6 month postimplantation, the implant had degraded and smooth corneal epithelial layer had been created.[361] Collagen films loaded with human growth hormone promoted wound healing in a mouse model,[362] while when loaded with etoposide, an anticancer drug, they were used in a liver model.[363] Tubular films, alone[360,364] or loaded with a neurotrophin-3-encoding gene,[365] have demonstrated increased axonal alignment, enhanced neovascularization, axonal regeneration and myelination in rat sciatic models. When these tubes were loaded with collagen fibers, guided Schwann cell migration, decreased axonal dispersion and reduced axonal mismatch in a rat sciatic nerve model were observed.[366] In a rabbit model, the dura was replaced with cyanamide crosslinked collagen films, which displayed very low inflammatory response and increased synthesis of new collagen by connective tissue cells that infiltrated the film by day 56 postimplantation.[156]

Collagen films wrapped in form of tube have been extensively used in clinic as nerve guidance conduits (e.g., NeuraWrap, NeuroMend, NeuroMatrix, and NeuraGen),[367] demonstrating limited myofibroblast infiltration, guided Schwann cell migration and axonal regrowth toward their distal targets.[368] Nonetheless, such materials are limited to nerve gaps smaller than 4 cm in length.[369] Tetracycline-immobilized crosslinked collagen films have been used clinically for treatment of periodontitis and have been shown to be successful in reducing the density of microorganisms.[370] Collagen calcium-alginate films have been used as wound dressing to treat burn patients, demonstrating significant increase in epithelialization, while patients experienced reduced pain levels.[371] Collagen type IV films have been implanted into patients suffering from tympanic pocket retraction and demonstrated complete healing 6 month postimplantation, a potential alternative to autologous tissue.[372] Collagen films have been implanted and assessed after transvesical prostatectomy, exhibiting no adverse reactions.[373] Despite the overall promising results in multiple clinical indications, the produced films are comprised of isotropic collagen fibrils that fail to imitate the hierarchical architecture of native tissues. To this end, various technologies have been utilized to produce biomimetic anisotropic collagen films.

Subjecting collagen solutions to a magnetic field during fibrillogenesis allows development of films with aligned subfibrillar structure.[294] Collagen fibrils align perpendicularly to the magnetic field due to their negative diamagnetic anisotropy of the α-chains.[374] In general, magnetic fields of 1.9–12 T are applied for 30–90 min.[375–379] Multilayer magnetically aligned collagen-proteoglycans based scaffolds have been used to align human keratocytes in culture,[375] while magnetically aligned collagen-hyaluronic acid scaffolds have been used to maintain primary chondrocytes in culture, albeit the addition of hyaluronic acid decreased the effectiveness of magnetic alignment.[376] In the neural space, magnetically aligned collagen has been shown to orientate Schwann cells and neurons in vitro[377,378] and to promote new nerve fascicle formation in a mouse sciatic nerve model.[378] It is worth pointing out that ribose-crosslinked magnetically aligned collagen scaffolds proved detrimental for regeneration.[378] Plastic compression has been incorporated into the fabrication process to increase mechanical properties and to reduce degradability, resulting in primary murine tenocyte alignment for up to 18 d in culture.[379] Given the high cost of the superconducting magnets required to induce alignment, the use of iron oxide particles has been proposed, as this method requires magnets of low strength (0.001 T).[380]

Given the complexity of the magnetic field induced alignment, microfabrication technologies have been adopted, which have facilitated the generation of structured collagen substrates with precise and reproducible topographical features with nano- and microscale resolution. Soft lithography refers to the replication of microfeatures on collagen materials using a patterned elastomeric stamp (**Figure 13**). Soft lithography has been used for replicating grooves, holes and pillars[381] and for the encapsulation of cells in single forms or multiarrays.[382] Collagen films, casted on poly(dimethyl siloxane) templates, induced bidirectional elongation of human vascular smooth muscle cells.[383] Collagen injection using microfluidics into sacrificial stamps or molds that precisely contain the structure to be reproduced has also been used as means to produce structures with features of a few microns capable of aligning cells.[384] Aligned collagen films have also been produced via molecular imprinting, through the generation of high and constant shear forces during the collagen deposition on glass substrates.[385–387] Shear force is applied by lateral displacement of the injection needle and orbital spin of the collector. The set of parameters depend on the method; lateral displacement requires thin syringe needles of about 18–27 gauge, lateral speed of 100 mm s^{-1} and collagen flow of 0.3 mL min^{-1} approximately for orienting collagen.[386,388] The orbital spin method requires high spinning rates of 500–3000 rpm and collagen flow of 0.3–1.0 mL min^{-1}.[387,389] Both molecular imprinting methods require fast collagen desiccation, less

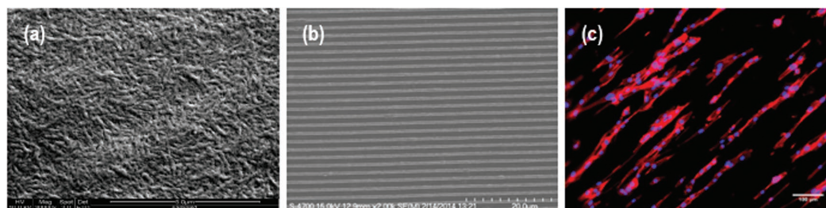

Figure 13. a) SEM analysis reveals the fibrous nature of collagen films. Through soft lithography, anisotropic collagen films can be produced (b), which induce bidirectional human skin fibroblast growth (c).

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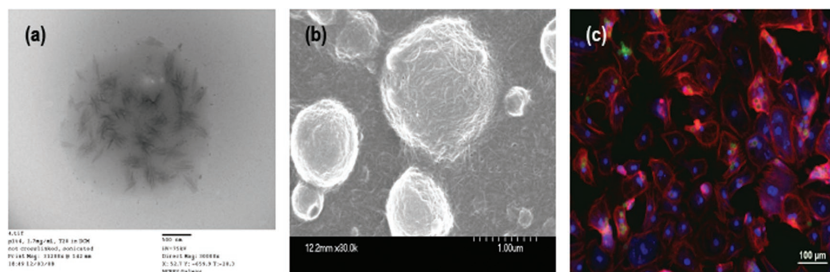

Figure 14. a) TEM and b) SEM analyses clearly illustrate the fibrous nature of hollow collagen microspheres. c) Fluorescent microscopy of primary human cardiac fibroblasts uptaking 10 µm FITC-labeled hollow collagen type I microspheres (red: rhodamine phalloidin; green: FITC-labeled spheres; blue: DAPI).

than 15 min, to stabilize the fibril structure and alignment. However, fibril orientation is not stable, fibrils often turn and as such, alignment is slightly altered. This difficulty can be partially solved using collagen at high concentration and reverse dialysis.[386,388] Given that recent data have questioned the potential of structured substrates for in vivo applications, we expect that such structured substrates will be primarily used for in vitro applications (maintain cell phenotype and direct stem cell lineage).[390]

6.6. Template-Produced Hollow Spheres

Hollow microspheres offer several advantages over other carrier systems for delivery of bioactive and therapeutic molecules including: reproducibility, large surface area, large cargo delivery capacity, controllable biodegradability, and multicargo delivery capacity.[391,392] In recent years, several methods including emulsion, spray-drying and microphase separation have been investigated for the development of collagen reservoir systems for sustained and localized delivery of drugs and biologics.[393] However, these techniques offer little control over reproducibility,[394] which triggered investigation into the template method.[394,395] With the template method, a natural polymer is deposited on the appropriate template, which afterward is removed, leaving behind the hollow polymeric shell.[391–396] Polymer-based templates are preferred, as they can be fabricated with controlled size, shape and dispersity.[391] Hollow collagen spheres (**Figure 14**) have been produced using sulphonated polystyrene beads as templates.[394] As the coating process is based on an electrostatic interaction between collagen and the negatively charged polystyrene template, polystyrene beads are sulphonated to impart a strong negative charge. The coating process is performed under acidic conditions so that the positively charged collagen forms a thin coat around the negatively charged polystyrene beads. Following sulphonation, the beads are resuspended in acetic acid and the collagen solution is added to the beads. After formation of the collagenous coating around the polystyrene bead, the collagen is crosslinked. Finally, the polystyrene core is removed with tetrahydrofuran, leaving behind the hollow collagen sphere. To date, such scaffolds have been used for gene,[394,397] growth factor,[398] and drug[399] delivery or ROS scavenging.[400]

6.7. Tissue Engineered Structures

Advancements in tissue engineering technologies have enabled the development of scaffold-free tissue engineering therapies,[401] during which a living substitute is formed that is held together with cell–cell and cell-deposited ECM contacts. Such approaches are based on the inherent capacity of cells to synthesize matrix.[402] Given that lysyl oxidase is a copper-dependent enzyme,[403] it has been suggested to add copper ions into the culture media to increase lysyl oxidase-mediated crosslinking (e.g., hydroxy pyridinoline and pyridinolines) for the mechanical improvement of tissue engineered arteries[404] and cartilage.[405] Although very promising preclinical and clinical data are available for various clinical indications, including skin,[406] blood vessel[407,408] and cornea,[409] only a handful of products have been commercialized (e.g., Epicel, Genzyme; LifeLine, and Cytograft). The substantial long culture time required to develop an implantable device (e.g., 70 d for lung cell sheet,[410] 84 d for corneal stromal,[411] and 196 d for blood vessel[408]) has been recognized as the major limitation for the wide acceptance of this technology. To remedy this, macromolecular crowding has been introduced as means to accelerate ECM deposition (**Figure 15**). In vivo cells reside in a highly crowded extracellular space, which results in rapid conversion of the de novo water-soluble procollagen to water insoluble collagen.[41] In the dilute culture media, this procollagen/collagen conversion is very slow. The addition of inert macromolecules into the culture media, by emulating the naturally crowded in vivo milieu, amplifies deposition of cell-secreted ECM.[412] Polydispersed macromolecules have been shown to be more effective with respect to ECM deposition, due to more efficient volume exclusion effect.[413] To date, macromolecular crowding has been shown to enhance ECM deposition in permanently differentiated cell culture[414] and in naïve stem cell culture[415] and to enhance adipogenesis in adipose-induced stem cell culture.[416] Macromolecular crowding has also been proposed as means to develop in vitro pathophysiology models.[417] Further, human fibroblast matrices, developed under macromolecular crowding conditions, have been shown to support stable propagation of human embryonic stem cells ex vivo.[418] Such system can be used as an alternative to Matrigel, a cell-produced material (murine in origin, derived from the Engelbreth–Holm–Swarm sarcoma cell line[93]) rich in laminin, collagen IV, heparin sulfate proteoglycans and a number of growth factors that has been used extensively for optimal ex vivo cell growth.[419]

7. Conclusions

It is evidenced that significant strides have been made in the field of collagen research. Advances in purification processes have made collagen preparations available with minimum immunogenicity/antigenicity (dilute acetic acid, pepsin digestion, filtration to remove impurities/insoluble matter, salt precipitation, and dialysis are crucial steps in the

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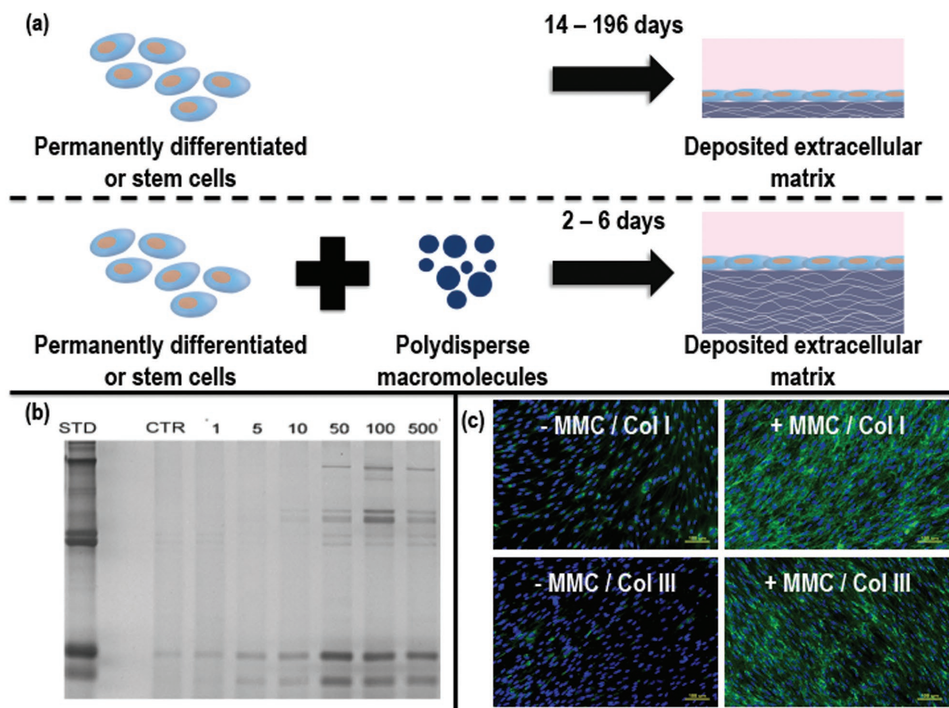

Figure 15. a) In normal permanently differentiated and stem cell culture, ECM deposition is very slow and as such the production of living substitutes can take up to 196 d. The addition of polydispersed macromolecules (macromolecular crowding) in culture media dramatically accelerates ECM deposition and living substitutes can be produced within 6 d in culture. b) SDS-PAGE analysis of human bone marrow mesenchymal stem cell layers demonstrates that ECM deposition is dramatically enhanced as a function of carrageenan concentration (1, 5, 10, 50, 100, and 500 µg mL^{-1}) after 2 d in culture. c) Immunocytochemistry analysis further corroborates the enhanced collagen type I and collagen type III deposition after 2 d in culture (cells: human bone marrow mesenchymal stem cells; macromolecular crowder (MMC): 100 µg mL^{-1} carrageenan).

production of high purity and low immunogenicity soluble collagen), while advancements in biotechnology and bioengineering have made available recombinant collagens and collagen-like peptides. Advances in chemistry have enabled the development of elegant crosslinking systems (e.g., branched PEG systems, plant extracts) that offer control over mechanical properties and degradation rate, do not trigger foreign body/immune response (crosslinking should be accompanied by extensive washing with distilled water, phosphate buffer saline, or glycine for favorable cell response), and enable the scaffold to interact with the host through the addition of a stimuli-responsive moieties. The evolution of engineering and nanotechnology enabled development of elegant/sophisticated controlled shape, porosity, and topography 3D hierarchical structures that offer opportunities for localized and sustained delivery of bioactive/therapeutic molecules and living cell populations. Bioinspired in vitro culture methods (e.g., macromolecular crowding) have enabled the development of ECM-rich living tissue substitutes and pathophysiology models for drug discovery purposes. As new tools and technologies are becoming continuously available, we predict that collagen will play a pivotal role in reparative medicine. For example, as the 3D printing technology improves, we anticipate seeing in the foreseeable future the development of patient-specific collagen-based implants. This prediction is based on the observation that the global collagen market expands at 9.4% compound annual growth rate and the market is projected to rise to US$9.37 billion by 2023.[420] It is evidenced that collagen, the oldest protein ever sequenced,[421] has still many tricks up its sleeve.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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