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Strategies for Directing the Structure and Function of 3D Collagen Biomaterials across Length Scales

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Abstract

Collagen type I is a widely used natural biomaterial that has found utility in a variety of biological and medical applications. Its well characterized structure and role as an extracellular matrix protein make it a highly relevant material for controlling cell function and mimicking tissue properties. Collagen type I is abundant in a number of tissues, and can be isolated as a purified protein. This review focuses on hydrogel biomaterials made by reconstituting collagen type I from a solubilized form, with an emphasis on in vitro studies in which collagen structure can be controlled. The hierarchical structure of collagen from the nanoscale to the macroscale is described, with an emphasis on how structure is related to function across scales. Methods of reconstituting collagen into hydrogel materials are presented, including molding of macroscopic constructs, creation of microscale modules, and electrospinning of nanoscale fibers. The modification of collagen biomaterials to achieve desired structures and functions is also addressed, with particular emphasis on mechanical control of collagen structure, creation of collagen composite materials, and crosslinking of collagenous matrices. Biomaterials scientists have made remarkable progress in rationally designing collagen-based biomaterials and in applying them to both the study of biology and for therapeutic benefit. This broad review illustrates recent examples of techniques used to control collagen structure, and to thereby direct its biological and mechanical functions.

Keywords

collagen; scaffold; fabrication; structure; biomaterial; tissue engineering

1.0 - Introduction: Collagen as a Biomaterial

The collagens comprise a superfamily of proteins that includes over 20 members of varying abundance, functionality, and distributions within tissues. The topic of this review is the fibrillar protein collagen type I, one of the most common structural elements in a variety of tissues and a widely used protein in the field of biomaterials. Its unique properties and relative abundance in living tissue have made it an appropriate choice in a variety of restorative applications throughout medical history, and more recently it has been a key material in tissue engineering and regenerative medicine.

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Two of the earliest papers to examine the structure of collagen focused on the spacing between repeating modules of the crystalline polypeptide chains of connective tissues like tendon, hair, and gut [1, 2]. Continuing studies of the basic crystalline structure of collagen led to elucidation of the primary structure in the 1950s, which facilitated a more fundamental understanding of the protein's mechanical and chemical properties. In 1954, Ramachandran described the hexagonal packing of the helical secondary structures in the collagen molecule [3]. Over the next two years Rich and Crick [4] and Ramachandran [5] revised and validated the proposed helical structure. A comprehensive history of the evolution of our understanding of collagen structure is provided in Beriso et al [6].

The current model of the basic unit of collagen (the "tropocollagen" molecule) consists of a helical quaternary structure called a Madras triple helix, which is composed of three left handed alpha helices. In the collagen type I isoform, two of the chains are identical and the third has a similar but distinct sequence. The alpha chains are composed of repeating motifs of three amino acids that typically have of a glycine at every third interval. Glycine is the smallest essential amino acid, and its presence in the chain allows the rotational freedom needed to form a helical structure. Proline and hydroxyproline (and less frequently hydroxylysine) are other important components of the helices that impart structural and functional features. Inter-chain covalent and hydrogen bonding between the residues of these amino acids imparts stability and rigidity to the molecule. The remaining cohort of amino acids on the collagen type I molecule have a variety of residues that impart biochemical and physical attributes important for cell attachment and other biological functions.

An important characteristic of collagen type I is its well understood hierarchical structure from the nanoscale to the macroscale, shown schematically in Figure 1. Cells internally synthesize, modify, and assemble the alpha chains into a procollagen form, which is secreted to the extracellular space and then partially cleaved by specific enzymes to form the tropocollagen molecule. These nanoscale subunits (typically about 1.5 nm in diameter and 300 nm in length) further self-assemble into fibrils that consist of multiple tropocollagen molecules, which can be tens to hundreds of nanometers in diameter and on the order of microns in length. The tropocollagen molecules are covalently bound to each other in a staggered manner, giving collagen fibrils a distinctive banded pattern when viewed at high magnification. Fibrils can then assemble into larger and longer fibers and fiber bundles on the order of microns to centimeters in scale, and such fibers are major structural components of many tissues (Figure 2). The intricate and highly organized architecture of collagen materials, from the level of alpha helices to fiber bundles, results in a stable extracellular matrix protein with high tensile strength.

The well understood structure and the remarkable biochemical and physical properties of collagen have made it a widely used biomaterial in a variety of applications. Collagen is an excellent attachment substrate for cells, which can recognize and bind to the protein through integrin receptors (reviewed in e.g. [7, 8]). Cells not only attach to collagen but can also degrade it by secreting specific enzymes and can synthesize new collagen via intracellular production and export to the extracellular space. In this way, cells remove, remodel, and replace collagen, a process that is important in the homeostasis of many tissues and organs. In addition to its biological relevance, a key advantage of this material is its abundance in tissues across species. Collagen type I accounts for over 25% of the protein mass in mammals, and it can be isolated in relatively large quantities using straightforward biochemical methods. These cellular interactions and the natural abundance have been exploited by biomaterials scientists to design, develop, and fabricate collagen-based materials and engineered tissues.

This review describes some of the strategies that have been used to create, control, and modify the structure and function of three-dimensional collagen-based biomaterials. In keeping with the theme of this special issue of Acta Biomaterialia, the emphasis is on how our understanding of the key biochemical and physical properties of collagen has been used in biomaterials design across length scales. Figure 1 schematically shows the structure of collagen type I across length scales, as well as how these structural features relate to the function, fabrication, and modification of collagen hydrogels. We examine strategies that can be applied to control the fabrication and remodeling of collagen materials, and thereby can affect their structure as well as biological and mechanical function. Due to the wide range of work in this area that dates back over 40 years, this review cannot be an exhaustive treatment of the subject. Rather, we highlight some relevant examples of how collagen structure and function have been manipulated for the purposes of biomaterials science. We attempt to cover some of the seminal contributions in this area, but concentrate on advances that have been made over the last decade.

We have focused this review on three-dimensional “reconstituted” collagen type I hydrogels; that is, materials that are formed from isolated collagen that is solubilized and then re-assembled under controlled conditions to generate desired three dimensional architectures and functions. A key advantage of these materials is that cells can be incorporated directly into the matrix at the time of fabrication, and therefore many of our examples highlight cell-containing systems. Our focus is not intended to downplay the importance of other forms of collagen materials such as foams, sponges, and dehydrated scaffolds, or the variety of decellularized native matrices recently developed. On the contrary, we recognize that these are among the most widely used biomaterials in regenerative medicine, and refer the reader to recent reviews on these materials [9, 10, 11, 12, 13]. In addition, we have limited the large majority of our analysis to *in vitro* efforts, because of the high variability and dynamic environment inherent in implantation of collagen materials. Therefore, our goal is to provide an overview of strategies that biomaterials scientists can use in the lab to direct the structure and function of cell-seeded collagen type I hydrogels. We hope that this review provides a starting resource for those interested in the topic, and the reader is encouraged to delve further into the cited primary literature for details. For those already well versed in the applications of protein-based hydrogels, we hope this review will provide a concise compilation of recent strategies for controlling collagen structure and function.

2.0 - Isolation and Reconstitution of Collagen into Hydrogel Materials

The recognition that collagen is an abundant and critical component of the extracellular matrix quickly led to an interest in its applications in biology and medicine. Collagenous substrates were used in cell culture as early as the 1930s and were quite widely investigated by the 1950s [14, 15, 16]. By the 1970s, the desire to mimic the extracellular matrix led to the use of isolated collagen in three dimensional cultures [17].

A key feature of isolated collagen is that it can be reconstituted into solid-phase materials and rehydrated to form robust hydrogels. For the purposes of this review, the hydrogel form is of most interest, because collagen in this form has been used widely as a biomaterial to emulate the extracellular matrix. A hydrogel is generally defined as a material that absorbs large quantities of water yet behaves as a solid. The solid phase of the material represents only a small fraction of the total mass and volume, but provides structure to the material in an aqueous environment. In the case of reconstituted collagen, the formed fibrils sequester water but resist swelling and dissolution of the material. The fibrils are physically enmeshed and in some cases covalently linked, providing resistance against swelling-induced tensile forces.

The basic method for isolating collagen type I from collagen-rich tissues such as tendon and dermis is straightforward, and involves breaking the tissue down via enzymatic digestion and/or dissolution in acid. The preferred process depends on the intended application and the desired characteristics of the collagen, and is different depending on the species [18, 19] and type of tissue [20, 21]. In these processes, collagen-rich tissues are immersed in either enzymatic or acidic solutions (or in some cases both) to degrade the tissue and release the protein into a solvent. The procedure varies with enzyme and acid strength, though typically several days are required to fully break down the tissue. The efficiency and results of the isolation process are dependent on the structure of the collagen. In particular, highly crosslinked tissues are more resistant to acid solubilization [22] and therefore enzymatic degradation is useful in liberating the collagen protein [23]. Enzymatic action cleaves the telopeptides on the ends of the collagen molecule that help stabilize the molecular structure, and therefore increases the efficiency of dissolution [24]. However, the lack of telopeptides on isolated collagen molecules has also been reported to alter its characteristics as a biomaterial [25, 26].

The general process for reconstituting dissolved collagen and thereby creating collagen hydrogel constructs has been used widely since the 1970s. Solubilized collagen is maintained at low pH and low temperature to prevent annealing of the dissolved peptide fragments. Raising the pH and temperature allows aggregation and covalent bonding of the collagen fragments to reform fibrils and create a hydrogel structure. Typically, solubilized collagen is poured into a mold and then exposed to a neutralizing agent, such as exposure to ammonia vapor to initiate fibrillogenesis [27]. More recently, sodium hydroxide solutions have been used for this purpose, and can be directly mixed with cold collagen solutions immediately prior to introduction into a mold. Subsequently raising the temperature initiates collagen fibrillogenesis and gelation of the construct. Importantly, these hydrogels can be made at physiological pH and temperatures, which allows incorporation of other biological components. Culture medium, serum, other protein constituents, and cells all have been incorporated into collagen hydrogels in efforts to mimic key aspects of the physiological environment. Varying the pH and temperature during gel formation has been shown to affect the structure of collagen gels. Lower pH leads to more compliant materials with decreased fibril diameter [28, 29], possibly due to protonation of the COOH groups and a consequent reduction in interactions between the carboxyl and amino acid groups [30]. Higher temperatures affect the structure of nascent collagen by providing energy to increase the rate of fibrillogenesis and decrease both the diameter of the fibers [31] and the pore size in the meshes [32], which can result in increased mechanical properties [33].

2.1 – Molding of Macroscopic Constructs

The straightforward method for reconstituting collagen hydrogels from solutions of isolated protein is an advantage when creating biomaterial constructs in a variety of shapes and sizes. It allows three dimensional hydrogel constructs to be fabricated via reproducible and flexible molding procedures in which the liquid collagen solution is introduced into a preformed template of desired geometry. This method probably offers the most direct approach to defining the macroscopic features of the biomaterial construct, and can be used to create large scale samples for both experimental and therapeutic application. Figures 3A [34] and 3B [9] show examples of macroscale constructs created in simple disk and tube shapes via molding and gelation.

A useful application of hydrogel molding is creating collagen constructs in geometries that facilitate application and/or measurement of macroscopic mechanical properties. For example, hydrogel constructs can be shaped into the “dogbone” geometry that is conventionally used for tensile properties measurement of a variety of materials [35, 36, 37]. This shape facilitates gripping of the material while also providing a consistent gauge

section. However, gripping of hydrated collagen materials without tearing them is challenging, and therefore collagen constructs can also be made as rings that can be suspended between stirrups for mechanical testing [38, 39, 40]. Alternately, the ability to "mold-in-place" can be used to create tight integration of collagen constructs with mechanical grips or actuators, for example by gelling the collagen solution around porous grips [41] or strategically placed posts (Fig 3C, D)[42]. Such a strategy can be used to measure cell-generated forces in three dimensional collagen hydrogels using micromolded cantilevers (Fig. 3E, F) [43]. In addition, geometries conducive to compressive testing are also easily fabricated, and usually are in the form of disks or cylindrical samples.

The variety of construct geometries described above can be used to determine conventional macroscopic sample and material properties such as stiffness, strength, and toughness, by analyzing uniaxial or multiaxial stress-strain data. However, collagen hydrogels typically exhibit viscoelastic properties due to their high water content and interaction between the solid and liquid phases. The ability to mold collagen also facilitates the creation of samples for viscoelastic testing. To this end, rheometric analysis has been performed to assess collagen gelation behavior [44, 45] and differences between material formulations [46, 47, 48], as well varying construct geometries and architectures [49].

The inclusion of a cellular component embedded in collagen hydrogels at the time of fabrication adds another means of control, as well as substantially more complexity, to the molding and subsequent remodeling processes. One of the most well characterized processes is the capability of embedded cells to exert forces on collagen fibers and to "compact" or "contract" three dimensional collagen hydrogels, as shown in Figure 4 [50, 51]. A variety of cell types exhibit this behavior, in which the volume of a three dimensional collagen gel is reduced through matrix densification and expulsion of water. This process requires force generation by the cells [52, 53], and often results in dramatic remodeling of the matrix. It is at least partially mediated by the matrix metalloproteinase class of proteolytic enzymes, since inhibition of these pathways has been shown to prevent gel compaction [54, 55, 56, 57]. Embedded cells also can remodel collagen to align the fibrils, typically in response to mechanical forces. When collagen gels are constrained from compacting freely, tensile stresses are generated as the cells pull on the fibrils. This effect has been explored by comparing mechanically constrained cell-seeded gels to similarly prepared unconstrained gels that have been completely released from the mold walls and allowed to compact freely [50, 58, 59]. Constraining the hydrogels results in marked differences macroscopically, and in cell and fiber alignment at the microscale. This phenomenon has been harnessed to create patterned and aligned fibrillar matrices, using molds with strategically placed constraints [60, 61] and posts [42, 51].

Control over the internal architecture of macroscopic collagen matrices can also be achieved using other methods applied during gel formation. Micropatterned molds have been used to create desired surface topographies and microstructures [62]. Microfluidic techniques have been used to create collagen matrices containing perfusable channels [63, 64, 65], gradients in collagen morphology [66], and to create stable interfaces within engineered tissues [67]. The degree of compaction of the matrix can also be externally influenced by applying plastic compression during or after gel formation [68, 69]. Therefore, there are a number of strategies that can be combined to define both the macroscale and microscale features of collagen gels, and which involve relatively straightforward manipulation of the collagen molding and gelation processes. While control over the matrix structure provides the ability to vary the functional properties of the material, it remains a challenge to independently vary structural properties. However molding of collagen hydrogels does provide facile control of a richly functional extracellular matrix protein and therefore offers advantages in creating biologically relevant biomaterials.

2.2 – Modular Microtissues

While the ability to create large tissue structures using collagen is valuable in some applications, there has been a growing trend to create smaller, modular microenvironments that can then be assembled into larger architectures [70, 71, 72]. One advantage of this approach is that modules may be made small enough to be injectable, providing the possibility of assembling larger-scale components while still using minimally invasive delivery. Another advantage is that multiple types of modules can be created separately, and can then be assembled into larger multiphase constructs that consist of multiple module types in defined architectures. Cells can be embedded within the modules at the time of fabrication and/or can also be seeded on the surface of the modules. The properties and composition of the modular units can be tailored to promote specific cell functions and differentiation into desired tissue types, though determination of the mechanical properties of micro-scale modules can be challenging.

Several approaches have been taken to create modular collagen-based biomaterials. Pure collagen modules have been generated using an extrusion process to create long collagen threads, which can then be cut into modular cylindrical units (Fig. 5A, B) [73]. Incorporation of stromal cells in the collagen matrix and subsequent coating of these microscale modules with endothelial cells has been shown to lead to assembly of larger vascularized organoids when these preparations are cultured under flow in vitro [74]. Similar collagen-based modules have been used to promote neovascularization in vivo [75, 76, 77], for cardiac applications [71], and for pancreatic islet transplantation [75]. In an alternate approach, pure collagen microspheres ranging from 1-3 mm in diameter have been produced by creating neutral collagen droplets and allowing them to gelate on a non-adhesive surface [78]. Pure collagen modules have also been used for cartilage tissue engineering [79], and creation of layered skin [80], and these collagen modules have been used to create multiphase tissue constructs containing different cell types [81]. A microfluidic technique has also been used to create cell-laden collagen microspheres [82], as well as acellular pure collagen microspheres, which were subsequently coated with cells and used to demonstrate assembly of monoculture and co-culture aggregates [83]. Such collagen modules have also been modified to enhance their desired properties, for example, by adding hydroxyapatite or chitosan [84, 85, 86, 87]. The modular format of these constructs provides a great deal of flexibility to create structures with defined architectures and multiple phases.

Collagen-based composites have also been used to create modular tissues. Emulsification of solubilized collagen and agarose in a hydrophobic phase can be used to produce highly spherical and uniform “microbeads” (100-300 μm in diameter) containing embedded cells [88, 89]. The fabrication process is similar in principle to creating macroscopic collagen constructs, and the composition of these microbeads can be altered to suit the application. For orthopedic tissue engineering purposes, collagen-chitosan materials have shown promise [90, 91, 92], and have also been used to create modular microbeads (Fig. 5C, D) [93]. This formulation has been used to embed cells and to create cohesive, multiphase macroscopic constructs composed of regions of aggregated microbeads in prescribed geometries (Fig. 5 E-G) [94]. Alginate has also been combined with collagen using an electrostatic dispersion method to create cell-containing beads 200-3000 μm in diameter for cardiac regeneration [95].

In an effort to achieve improved resolution and more precise control over construct organization at the microscale, a variety of fabrication techniques have been developed. Bioprinting and layering of collagen and cells has been investigated to create tissue structures resembling skin [96, 97] and cartilage [98]. These methods can now be used with materials of varying properties and are able to maintain cell viability [99]. Other

mechanisms based on the molecular interactions of collagen molecules in solution also have been applied to create ordered and well defined materials at the microscale [100, 101].

2.3 – Electrospinning of Collagen

The fibrillar nature of *in vivo* collagen has resulted in a variety of efforts to mimic this element of its structure. Electrospinning is a technique that has been used for decades to create polymer fibers, and which more recently has been applied to fabricating meshlike biomaterials [102, 103]. The principle involves applying an electric field to draw out a fine thread of charged polymer solution, such that the solvent evaporates and a solid fiber is formed. Typically, the polymer solution is ejected from a syringe and is drawn toward a charged collection plate or drum, where it is collected as a fine polymer strand often on the order of tens to hundreds of nanometers in diameter. Whipping of the strand or spinning of the collection drum allows collection of large quantities of the thread in a meshlike configuration, as shown in Figure 6 [110, 118]. Controlled collection can be used to create aligned or patterned meshes, and sheets of mesh can be layered to produce collagen constructs with different phases and corresponding properties [104, 105].

Collagen has been used in electrospinning for over a decade [106, 107], but because of its protein nature and susceptibility to denaturation, it has not been widely used in its pure form in this application. Typically, volatile organic solvents are used for electrospinning, which can alter the conformation of the dissolved collagen peptides [108] and prevent reassembly into fibrils that exhibit the native staggered configuration and distinctive banding pattern. Such malformed fibers are not stable in water and will dissolve over time [109, 112]. In addition, the toxicity and high salt content of the solvents conventionally used in electrospinning have hampered its use in cell-contacting situations. However, more recent efforts have resulted in the ability to electrospin collagen from relatively benign solvents, such as mixtures of ethanol and water [110], and crosslinking strategies have been developed to enhance the stability of even very thin fibers [111, 112]. These resulting fiber meshes are not hydrogels themselves, however, they are typically hydrated for use with cells, and the resulting matrices resemble fibrillar hydrogels.

The difficulty in electrospinning pure collagen has resulted in the use of composite materials that contain both collagen and a supporting material to produce biomimetic fibers. Polyethylene oxide (PEO) has been shown to facilitate electrospinning of natural materials, and has been used as an additive to collagen [106]. Composites of collagen and other extracellular matrix components including elastin [113, 114] and hyaluronan [115] have also been electrospun, though in these cases the resulting material is a blend of the components, rather than a mix of fiber types. Other naturally-derived materials such as silk [116, 117], chitosan [118, 119] and hydroxyapatite [120, 121] also have been combined with collagen for electrospinning. Similarly, synthetic polymers have been used to stabilize composite fibers containing collagen, though in this case organic solvents are typically required. A range of polymers have been used in this capacity, including polycaprolactone [122], polylactide-polyglycolide [123], polyurethanes [124], as well as blends of these materials [125]. Electrospun fibers containing significant fractions of collagen or other proteins typically also require crosslinking to maintain their stability [111, 126, 127]. While the fibrillar structures formed by electrospinning of pure and composite collagen materials have been shown to generally be better substrates for cell adhesion than purely synthetic materials, they typically lack the full functionality of native collagen.

3.0 - Modifying the Properties of Collagen Biomaterials

In addition to specifying the structure of collagen-based biomaterials at multiple scales via controlled fabrication, their properties can be affected by other strategies that directly target

the composition and morphology of the material. These treatments can be application-specific in that the collagen is modified or augmented to exhibit key features of specific tissue types.

3.1 - Mechanical Control of Collagen Structure

The mechanical environment in load-bearing tissues is complex and dynamic, and collagen's role as an important structural element in many of these tissues suggests a link between mechanical forces and collagen structure. Cells are not only the site of biochemical synthesis of the collagen molecule, but are also physically involved in guiding fibril formation and the development of larger structures [128, 129, 130, 131]. The response to mechanical strain of both the collagen matrix, as well as cells embedded in such matrices, has been exploited to enhance the structure and function of collagen biomaterials.

Tensile strain has been used widely to direct collagen assembly and remodeling (Fig. 7A) [41]. Uniaxial strain has been shown to induce alignment of cells and matrix parallel to the direction of tensile force (Fig. 7B, C) [41, 132]. Similarly, cyclic circumferential stretch of smooth muscle cell-seeded tubular collagen hydrogels has the effect of aligning both the cell and matrix components in a direction to resist the stress, and thereby increases the mechanical properties of the constructs [38, 133]. These effects are presumed to be cell-mediated, since cells sense forces through their binding interactions with the collagen molecule, and it has been observed that physiological levels of strain do not induce fiber alignment in acellular gels [134]. However, application of high levels of strain (30-50%) has been used to align acellular collagen constructs, with concomitant decreases in fibril diameter and increases in the tensile mechanical properties of the constructs [135].

Compression of cell-seeded collagen hydrogels also has impact on collagen architecture and subsequent function. Sustained compression compacts the gel and leads to structures with higher density [136], increased fiber diameter, and decreased pore size [137]. In this plastically compressed system, the cellular component is not required to achieve fiber reorganization and the resultant increases in mechanical properties [138]. Efforts to model these interactions and their effects have provided insight into both the mechanism of alignment [139, 140] and their effects on construct mechanical properties [141]. The interplay between applied mechanical forces, cellular responses, and the resulting remodeling of three dimensional collagen matrices is complex, but offers a powerful toolbox of techniques to modulate structure.

Fluid flow and the shear stresses it produces can also be used to direct the formation and remodeling of collagen matrices. Fibroblasts in three dimensional collagen gels respond to low (interstitial) flow rates by aligning perpendicular to the flow direction, and the matrix is subsequently realigned in the same direction [142]. The aligning effect of flow is also seen to a smaller extent in acellular collagen gels, suggesting that matrix-mediated contact guidance is also the mechanism for flow-induced cellular alignment [143]. Flow and mechanical strain have been combined in three dimensional collagen hydrogels [144] and can have both distinct and interacting effects on an embedded cellular component [145].

Microfluidic approaches offer excellent spatial control over creation of collagen architectures, and have been used to make graded and multiphase collagen constructs. Guided *in situ* collagen fiber assembly can be harnessed to create stable interfaces between distinct collagen gel phases [67]. A similar method has been applied to create local variations in collagen fiber size and density by taking advantage of the effects of temperature and protein concentration on collagen matrix assembly. This approach has used to fabricate graded multiphase engineered tissues [66]. Gradients in mechanical properties

also have been created in collagen hydrogels by carefully controlling the infusion of crosslinking agent using microfluidic techniques [146].

Tropocollagen is a dipolar molecule and its property of self-assembly results in a fibril with a permanent dipole moment. This physicochemical feature of collagen has been harnessed to magnetically align nascent fibrils perpendicular to the magnetic field direction as they are forming [147]. Magnetic alignment also has been used to orient the fibers in three dimensional collagen gels for the purpose of providing guidance cues to cells [148] and it has been demonstrated that the extent of alignment corresponds to the length of exposure (Fig. 7D, E) [149]. Magnetic collagen alignment increases the mechanical properties of the materials, however the field strength required to obtain oriented gels is high, generally above 6 T. Magnetic beads have also been incorporated into collagen solutions, so that they have a physical action on the gel during fibrillogenesis. In this case, simple bar magnets placed adjacent to the forming gels can induce alignment of the protein fibers [150, 151].

3.2 - Composite Collagen Hydrogels

The rationale behind creating composite materials is to combine two or more distinct materials to yield a new material with enhanced properties and function. This strategy has been applied to collagen type I in a variety of ways. This section focuses on the creation of “mixed” composites, which consist of materials that are homogeneous at the macroscale but which may have separate phases at the microscale (Figure 8) [92, 114, 152], as opposed to “laminar” composites that are created by combining macroscopic layers of separate materials. Collagen hydrogels are amenable to this approach because other materials can be added during gel fabrication and become an integral part of the matrix. Such composites can be designed to mimic desired elements of native tissue function, since all tissues can essentially be viewed as composite hydrogels.

An intuitive approach to collagen-based composite design is to augment the matrix with other extracellular matrix components that are also found in native tissues. Elastin has been used in this way [153, 154] to modulate the mechanical properties and recoil properties of the matrix [155, 156, 157]. Hyaluronan is an important component of many mammalian tissues, and similarly has been incorporated into collagen hydrogel materials [158, 159, 160] to enhance the sequestration of water and to provide compressive resistance. Other extracellular matrix proteins also have been used to augment collagen, including fibronectin [161], laminin [162, 163], Matrigel® [164, 165] (a mixture of basement membrane proteins), and other isoforms of collagen [166].

The clotting protein fibrin has been used in combination with collagen [167, 168], and is of particular interest as a biomaterial because of its relative abundance, as well as its remarkable biochemical and mechanical properties [169, 170]. While collagen type I is reconstituted using a pH and temperature-dependent mechanism, the insoluble fibrin protein is formed from its soluble precursor, fibrinogen, through enzymatic cleavage. This allows collagen and fibrin fibrils to be formed separately, creating interpenetrating fibrillar networks that result in robust hydrogels, either with or without an added cellular component. Varying the concentration and ratio of collagen:fibrin, as well as the type and concentration of enzyme used to initiate fibrillogenesis has been shown to modulate the microstructure [171, 172], macrostructure [39], and mechanical properties [40, 172] of collagen-fibrin composite matrices. In addition, the presence of fibrin in collagen biomaterials affects cell signaling and gene expression [173, 174], and therefore careful design of these and similar matrices can be used to promote desired tissue-specific functions.

Composites of collagen with synthetic polymers have also been investigated. Polyethylene glycol (PEG) can be used in hydrogel form, and has been combined with collagen to

produce composites either by physical entanglement [36] or by covalent bonding of polymers [175, 176]. Such materials have been shown to have enhanced interactions with cells, relative to PEG alone [177]. Degradable polyesters such as polylactic acid [178], polyglycolic acid [179], and polycaprolactone [180] are used widely as biomaterials, particularly in the area of regenerative medicine. However they do not form true hydrogels and their use in conjunction with collagen typically involves enhancing the surface or filling scaffold pores with protein to create a more cell-adhesive environment, or they are used as raw materials for electrospinning as described above. Another approach has been to create collagen-mimetic peptides that have defined sequence to promote self assembly, cell-adhesion, or other desired characteristics [181, 182]. These peptides have been immobilized to biomaterial surfaces [183] and have been conjugated to synthetic polymers to create hydrogels [184, 185, 186].

Addition of particulates to collagen materials to create composites has been widely explored to add strength and functionality to the matrix. For orthopaedic applications, calcium phosphate particles are a logical additive because of the mineralized nature of bone. Hydroxyapatite or tricalcium phosphate in micro- or nano-particulate form are often used in this application because they mimic the specific inorganic component of bone tissue. These additives are used less commonly in hydrogels but are widely used to augment freeze dried or electrospun collagen scaffolds (reviewed in Holzwarth et al. [187]). Hydrogel composites containing hydroxyapatite have shown enhanced cell adhesion and proliferation and collagen-hydroxyapatite formulations injectable through a syringe have been developed [188, 189]. Collagen has been shown to serve as the nucleation site for hydroxyapatite [190], and collagen matrices have been mineralized by immersion in simulated body fluid with high calcium and phosphate concentrations[188, 191]. Mineralization has also been potentiated by adding additional nucleation centers, including bioactive glass particles [192] and polymer spheres [193].

Other additives have also been investigated for augmenting the properties of collagen materials. In addition to mineral nanoparticles, collagen matrices have been doped with carbon [194], silver [195] and gold [196] nanoparticles. Carbon nanotubes are of particular interest because they are on the same size scale as collagen fibrils, with diameters on the order of nanometers and lengths on the order of microns. Hydrogel composites of collagen type I and carbon nanotubes can be fabricated by dispersing the nanotubes in dissolved collagen at the time of molding [197]. However, proper dispersion of relatively hydrophobic nanotubes can be problematic, and a range of functionalization and wrapping strategies have been used to promote interactions of nanotubes with water and proteins [198, 199]. Integration of carbon nanotubes into collagen hydrogels has been shown to increase the mechanical properties [200] and electrical conductivity of these composite materials [41, 201]. While synthetic nanoparticles offer the potential to impart new functionality to collagen materials, a better understanding of the physicochemical and biological properties of this very broad class of materials is needed in order to rationally design improved biomaterials. Cellular, tissue, and systemic toxicity of nanoparticules is a vibrant area of study and promises to illuminate the appropriate uses of nanoparticles in biological and medical applications [202, 203].

3.3 - Crosslinking Collagen Matrices

Native collagen contains both intramolecular and intermolecular crosslinks that play an important role in its structure and stability. In native tissues, these bonds form primarily through the action of the enzyme lysyl oxidase, which creates covalent links between lysine and hydroxylysine residues. Reconstituted collagen is stable in aqueous media, but the hydrogel form typically lacks the mechanical properties of native collagenous tissues due to both a reduced protein density and incomplete molecular crosslinking. Lysyl oxidase-

mediated crosslinking has therefore been applied ex vivo to increase the mechanical strength of collagen gels [204]. Non-enzymatic glycosylation, also called glycation, is the process by which collagen crosslinks are initiated by sugars. While formation of unwanted or excessive crosslinks is a hallmark of aging and certain pathologies, the crosslinking process can be harnessed to modulate the properties of collagen matrices. To this end, induced glycation has also been used to improve the mechanical properties of collagen hydrogel materials [205, 206].

Carbodiimides have been used extensively to crosslink collagen materials and to immobilize other proteins to the matrix. In particular, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is frequently used as a water-soluble crosslinker that forms zero-length bonds directly between collagen molecules. EDC crosslinking increases the mechanical properties of collagen hydrogels [152, 207, 208] and also increases their resistance to both hydrolytic [209] and enzymatic [210] degradation. Application of a braided architecture and EDC crosslinking has resulted in collagen scaffolds with mechanical properties similar to native ligament tissue [211]. EDC-treated collagen materials have been used extensively in the presence of cells [212, 213], and a variety of studies have suggested that residual toxicity is not a concern. Control over the degree of EDC crosslinking can be provided by modulating the time of exposure as well as by adding ethanol to the reaction [214].

Glutaraldehyde is a widely used crosslinker for biological tissues because it can react with several functional groups on proteins, and it has been applied as a stabilizer to collagen-based materials [215, 216, 217, 218]. Although gluteraldehyde is used in collagen-based bioprosthetic implants, it has has been associated with residual cytotoxity in vitro [219] and calcification in vivo [220]. Glyoxal is a much smaller aldehyde that can also crosslink collagen. It is a metabolite in the glycation process of proteins and is therefore found in low levels physiologically, but has not been investigated widely to modify biomaterials. Importantly, when used at low concentrations and limited times, glyoxal crosslinking of collagen materials can be accomplished in the presence of cells [92]. The plant-derived molecule genipin has also been used to crosslink collagen gels. Like other crosslinkers, the degree of crosslinking can be varied by controlling the duration, concentration, and pH of incubation [221, 222]. These treated constructs exhibit increased mechanical properties [90, 146] and have been reported to exhibit lower residual cytoxicity and inflammatory response than glutaraldehyde-treated materials [223, 224]. However, it has also been reported that crosslinking with genipin changes collagen matrix structure, including decreasing porosity [90*], and altering fiber structure and alignment [*225, 226*]*.

Exposure to radiant energy can be used to physically crosslink collagen materials. Photochemical crosslinking is achieved by shining visible or ultraviolet light through a sample in the presence of a photoinitiator, and has been shown to stabilize collagen gels and improve their mechanical properties and resistance to degradation [215, 227]. Gamma radiation also has been used for this purpose, with similar effects [228]. Crosslinking using external radiation has the advantage that the dose and time of exposure can easily be modulated, and the effect on collagen properties can be greater than achieved by chemical crosslinking [229]. Application of heat and resulting drying of collagen hydrogels, which is referred to as dehydrothermal crosslinking, is another technique used to create physical crosslinks. This method also alters the mechanical properties of the collagen materials [230, 231], though in this case cells cannot be included in the matrix at the time of fabrication.

4.0 - Summary and Perspectives

The role of collagen type I as a ubiquitous extracellular matrix protein and key structural element in native tissues has translated to broad and intense interest in its use as a

biomaterial. The protein's elegant and well understood hierarchical structure provides the blueprint from which new material architectures can be created. Biomaterials scientists have capitalized on our knowledge of collagen's composition and biological role to create a wide variety of material systems for examining biological questions, as well as for developing next-generation therapies.

At the nanoscale, collagen's primary structure results in helical self assembly of tropocollagen subunits to create robust molecular building blocks, which are further assembled into the microscale fibrils that are a fundamental component of the extracellular matrix of many tissues. The production of these fibrils can be controlled to regulate their diameter, length, density, and orientation. The degree of crosslinking within and between fibrils is also variable and can be used to further regulate the biological and mechanical properties of collagen materials. In both native and engineered tissues, association of collagen with other proteins and extracellular matrix components is another important determinant of the structure and function of the matrix. At the macroscale, the organization of collagen fibrils into larger fibers and fiber bundles is a key feature of the structure of many tissues. Taken together, these elements of collagen self assembly and organization offer a rich set of variables that can be manipulated to create biomaterials with desired architectures.

Biomaterials scientists have applied a range of biological, chemical, electromagnetic, and mechanical techniques to modify collagen structure and function. At the macroscale, molding of constructs and mechanical stimulation of matrix remodeling offer potent and relatively straightforward control over the shape and organization of bulk collagen hydrogels. The ability to create large protein constructs with biologically relevant architecture is a key advantage of using collagen as a biomaterial. Recent efforts to create modular microscale constructs made from collagen and composites with other materials have led to approaches to fabricate multiphase tissues that recreate more complex tissue architectures. Electrospinning and exogenous crosslinking of collagenous matrices offer nanoscale-level control over matrix dimensions and protein interactions. The ability to design and fabricate collagen structure at multiple scales makes it a versatile and effective biomaterial for obtaining desired biological and mechanical functions.

Collagen matrices are one of the oldest and most well understood classes of biomaterials. Early efforts to simply harness isolated extracellular matrix as a substrate for culturing cells have expanded into a wide range of techniques to capitalize on the protein's unique properties to create biomimetic hydrogels with well defined properties. Collagen materials are now used widely to study cell-matrix interactions and to create 3D microenvironments for the culture and directed differentiation of many cell types. Advanced techniques for controlling collagen architectures have made it possible to carry out experiments that illuminate cell function in tissues. They also have contributed to new rationally designed materials targeted at specific applications. These materials and the knowledge they generate are enabling the development of new cell- and matrix-based therapies that will overcome the limitations of less biologically relevant biomaterials.

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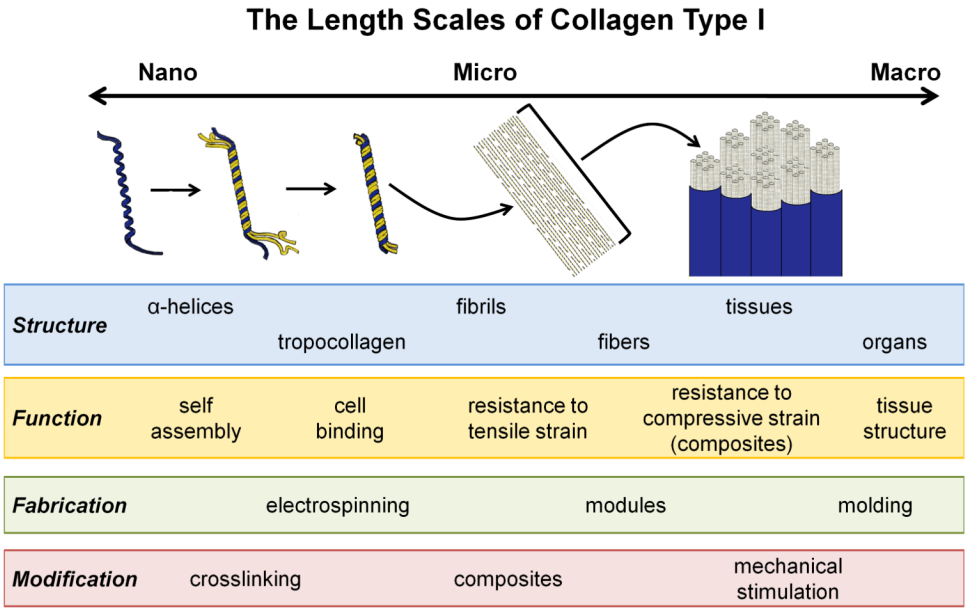

Figure 1.
The hierarchical structure of collagen type I leads to specific biological functions and characteristics across length scales. Fabrication of collagen hydrogel architectures can further be modified for particular applications.

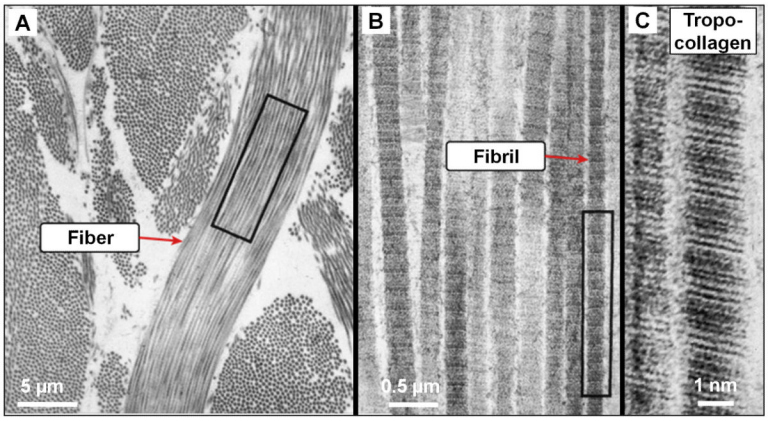

Figure 2.
Transmission electron micrographs showing the organized and hierarchical structure of the collagen molecule at the level of (A) collagen fibers, (B) collagen fibrils, and (C) the tropocollagen molecule. Scale bars are approximate. Adapted from [232].

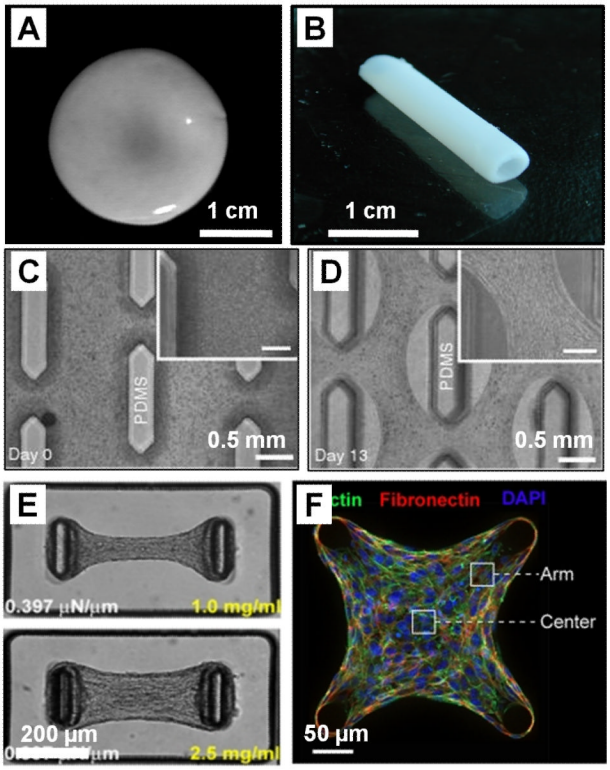

Figure 3.
Examples of molded collagen constructs. A) and B) show disk- and tube-shaped macroscale constructs, respectively, containing smooth muscle cells. C) and D) show cardiomyocyte-seeded constructs shaped around posts at day 0 and 13, respectively. E) and F) show fibroblast-seeded collagen constructs molded around cantilevers for force measurement. Cells in panel F are stained to show ECM and cellular components. Scale bars are approximate. Adapted from A) [34], B) [9], C and D) [42], E and F) [43]. Used with permission.

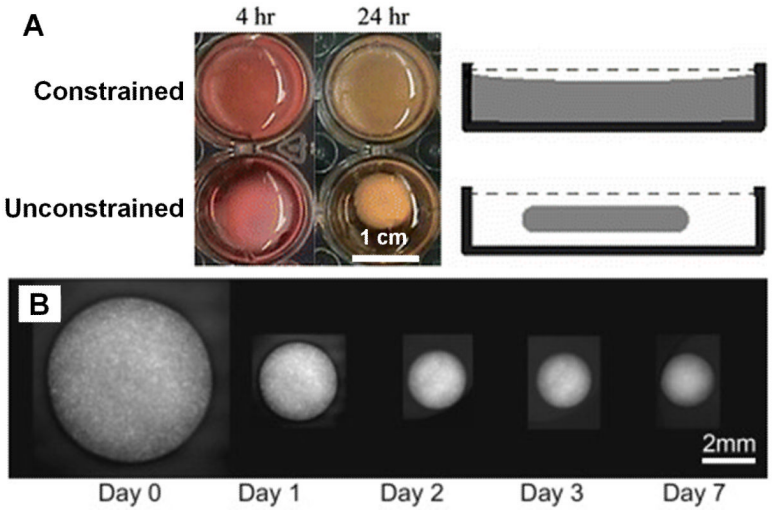

Figure 4.
Examples of gel compaction in A) constrained and unconstrained collagen hydrogels containing cardiac fibroblasts, and B) in a skeletal myoblast-seeded collagen hydrogel over time in culture. Scale bars are approximate. Adapted from A) [50], B) [51]. Used with permission.

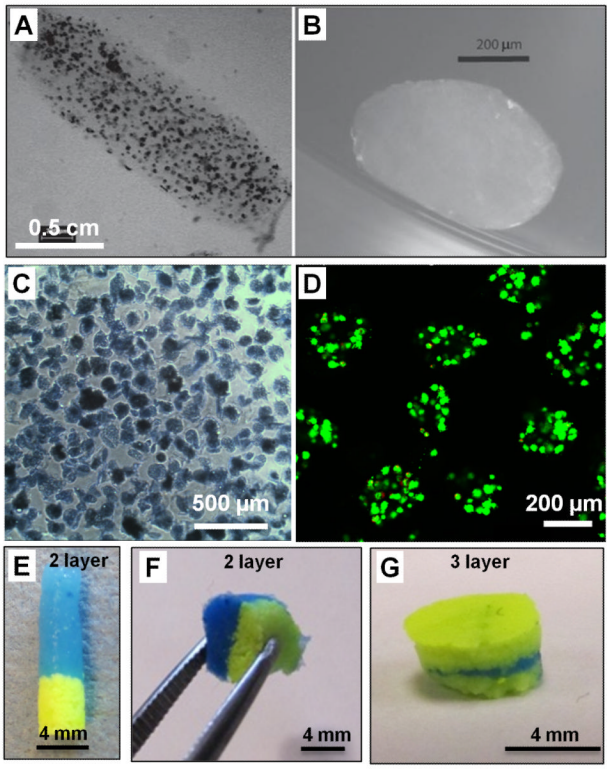

Figure 5.
Examples of modular collagen microtissues. A) and B) show collagen tissue modules with seeded endothelial cells. C) shows a light micrograph of collagen-fibrin microbeads stained blue to show protein content, and D) shows mesenchymal stem cells embedded in collagen-chitosan microbeads and stained green to show viability. E), F) and G) show larger scale constructs produced by assembling collagen-chitosan microbead preparations (microbeads stained blue and yellow for contrast). Scale bars are approximate. Adapted from A) and B) [73], C) [188], D) [93], E-G) [94]. Used with permission.

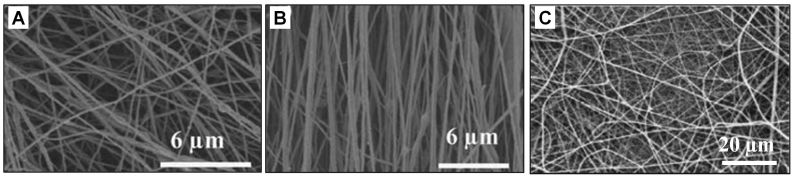

Figure 6.
SEM images of examples of electrospun collagen-based fibers. A) and B) show meshlike and aligned pure collagen fibers, respectively. C) shows a 50-50 collagen-chitosan fiber mesh. Scale bars are approximate. Adapted from A and B) [110], C) [118]. Used with permission.

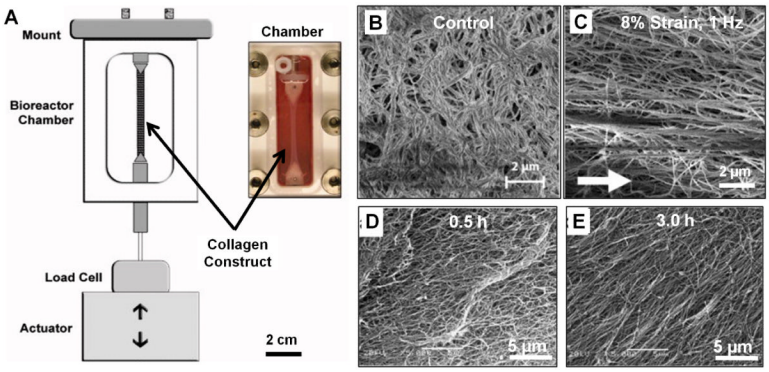

Figure 7.

A) Schematic and image of apparatus used for uniaxial cyclic strain to a fibroblast-seeded collagen construct. B) and C) show SEM images of unstrained and strained collagen matrices, respectively. D) and E) show collagen matrices that have been aligned by exposure to a 12T magnetic field for 0.5 and 3 h, respectively. Scale bars are approximate. Adapted from A-C) [41], D and E) [149]. Used with permission.

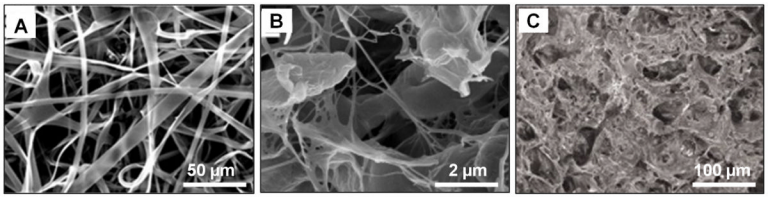

Figure 8.
SEM images of examples of collagen composite materials. A) shows electrospun 50-50 collagen-elastin fibers. B) shows a 50-50 collagen-chitosan molded material. C) shows an 80-20 silk fibroin-collagen molded material. Scale bars are approximate. Adapted from A) [114], B) [92], C) [152]. Used with permission.