Tissue Engineering: An Alternative to Repair Cartilage

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TISSUE ENGINEERING: AND ALTERNATIVE TO REPAIR CARTILAGE

Yaima Campos[a,b], Amisel Almirall[a], Gastón Fuentes[a,b], Hans L. Bloem[b], Eric L. Kaijzel[b] and Luis J. Cruz[b,*]

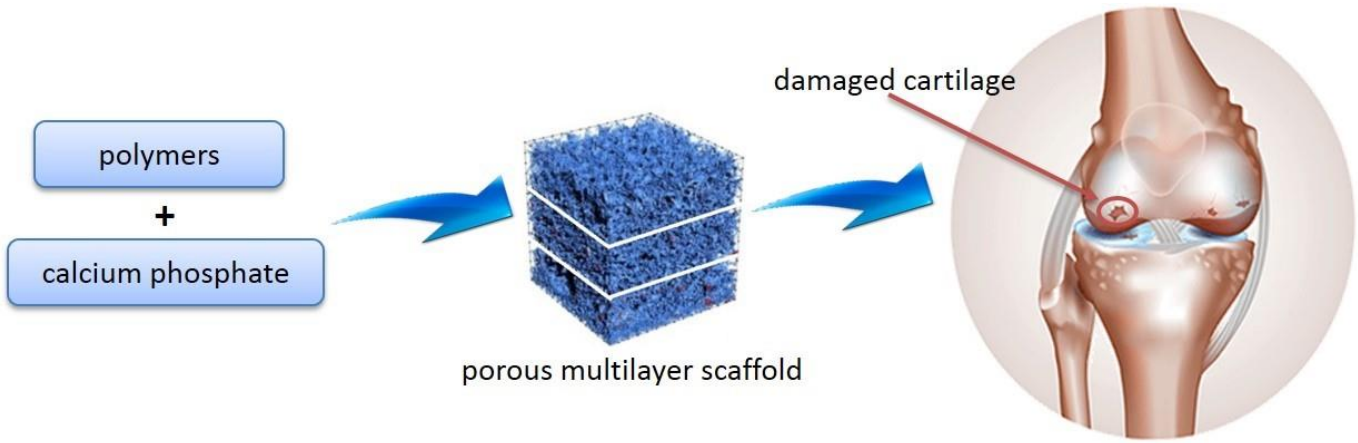
[a] Biomaterials Center, Havana University, Avenida Universidad e/ G y Ronda, Vedado, Plaza, CP 10400, LA Habana, Cuba

[b] Translational Nanobiomaterials and Imaging, Department of Radiology, Leiden University Medical Center, Albinusdreef 2, 2333ZA Leiden, The Netherlands

ABSTRACT

Herein we review the state-of-the-art in tissue engineering for repair of articular cartilage. Firstly, we describe the molecular, cellular and histologic structure and function of endogenous cartilage, focusing on chondrocytes, collagens, extracellular matrix (ECM) and proteoglycans. We then explore *in vitro* cell culture on scaffolds, discussing the difficulties involved in maintaining or obtaining a chondrocytic phenotype. Next, we discuss the diverse compounds and designs used for these scaffolds, including natural and synthetic biomaterials and porous, fibrous and multilayer architectures. We then report on the mechanical properties of different cell-loaded scaffolds, and the success of these scaffolds following *in vivo* implantation in small animals, in terms of generating tissue that structurally and functionally resembles native tissue. Lastly, we highlight future trends in this field. We conclude that, despite major technical advances made over the past 15 years, and continually improving results in cartilage repair experiments in animals, the development of clinically useful implants for regeneration of articular cartilage remains a challenge.

GRAPHICAL ABSTRACT



Key words: Articular, Cartilage, Tissue Engineering, Multilayer Scaffolds

Summary under 100 words: The cartilaginous tissue is very difficult to repair or regenerate due to its lack of vascularization and its small amount of cells. It is a very important biological tissue due to it is vital for the lubrication of the joints, which promotes a better and more organic movement of the body. This review aims to check the state of art of the the latest and most innovative trends in the biomaterials field for this tissue which are the three-dimensional multilayer structures that simulate the behavior under cyclic stresses of the articular cartilage.

INTRODUCTION

The main function of cartilage is to keep joints lubricated to ensure a smooth surface, which facilitates transmission of mechanical loads by providing the lowest friction coefficient possible. Unlike most tissue types, cartilage lacks blood vessels and lymphatic vessels and has a low cell population, which chiefly comprises chondrocytes. Consequently, endogenous cartilage recovery is slow and is limited by weak local transport of nutrients. Minor cartilage defects are currently repaired through medical interventions such as multiple drilling, abrasion arthroplasty, mosaicplasty (autologous osteochondral grafts) and cellular transplantation (namely, of autogenous and allogenic chondrocytes).[1] However, these techniques have their shortcomings. For instance, allografts can lead to transmission of diseases or provoke immunologic responses in the recipient, and once in place, remodel very slowly; and autografts demand that patients undergo several surgeries.[2]

An attractive alternative for cartilage repair is tissue engineering, whose testing and optimization over the past two decades has yielded many promising cartilage grafts and sophisticated bioreactor systems for *ex vivo* graft culture.[3-8] Modern tissue engineering comprises three basic elements: cells (chondrocytes, stem or progenitor cells), biodegradable scaffolds and growth factors. This approach is particularly amenable to restoration of articular cartilage,[9] as the scaffolds provide a three-dimensional network for chondrocyte growth[10] and act as mediators for cell-cell signaling and interactions. However, the physical and biochemical properties of these scaffolds are crucial.[11]

The aim of this article is to review the state of the art of three-dimensional scaffolds for the repair / regeneration of cartilage tissue explaining the composition of the tissue and the components currently used to prepare multilayer scaffolds by electrospinning, freeze-drying, etc.

COMPOSITION AND STRUCTURE OF CARTILAGE

Articular cartilage comprises a *non-mineralized layer* and a *calcified layer*; thus, it demonstrates inhomogeneous behavior. The non-mineralized layer has three structurally contiguous zones, each differing in matrix composition, organization and chondrocyte phenotype.[12-14] Briefly, the *surface zone*, located at the articulating region of the cartilage, accounts for approximately 10% of the total tissue height and consists of thin, elliptical chondrocytes and progenitor cells surrounded by a matrix with high water content (~78%), relatively low proteoglycan content, and collagen fibrils (diameter: 4 nm to 12 nm) oriented along the surface direction.[15] The cells in this zone produce surface protein area which contributes to joint lubrication.[16,17] Below the surface, the next 60% of cartilage depth corresponds to the *middle zone*, in which spherical chondrocytes reside within a matrix rich in proteoglycans and unaligned

collagen fibers (diameter: 9 nm to 60 nm).[18] Next, the *deep zone* of the non-mineralized layer corresponds to the final 30% of cartilage depth and is marked by spherical chondrocytes oriented in stacks perpendicular to the joint surface. These cells, while sparsely distributed, are within a matrix of relatively high glycosaminoglycan (GAG) content, high water content (~68%) and radially-oriented collagen fibrils (diameter: 60 nm to 140 nm).[19-23] Although the collagen fibril diameter generally increases from the surface zone to the deep zone, fine fibrils (<100 Å) have been observed to reside at all depths.

The calcified layer is situated between the deep zone of the non-mineralized layer, and subchondral bone.[24,25] It comprises hypertrophic chondrocytes located in a mineralized matrix that is rich in type I collagen and proteoglycans. This osteochondral interface ranges in thickness from 20 μm to 243 μm, a range whose breadth has been attributed to variability by age and by total cartilage thickness (Figure 1).[14,26,27] The levels of type II collagen and water decrease from the surface zone of the non-mineralized layer to the calcified zone, whereas the concentration of proteoglycans increases. The structural heterogeneity of cartilage leads to local variations in mechanical compressive module. This structural complexity underpins the challenges that researchers face in designing and assembling artificial scaffolds for cartilage repair.[25,28-32]

Main components of cartilage tissue

Articular cartilage, which is about 2 mm to 3 mm thick, has a complex structure that enables dissipation of mechanical loads (1 MPa to 4 MPa)[33] exerted on, and protection of, subchondral bone.[34] The extracellular matrix (ECM) chiefly comprises water (70% to 80%) and includes type II collagen and, to a lesser extent, glycoproteins (proteoglycans; Table 1). The type II collagen and glycoproteins, which attract water due to their negative charges, promote resistance to shear and tensile forces. The principal type of cell in this matrix is chondrocytes. However, given their low concentration (only 14 000 cells/μL to 30 000 cells/μL), they are limited in their growth, proliferation and, consequently, their capacity to repair collagen.[35]

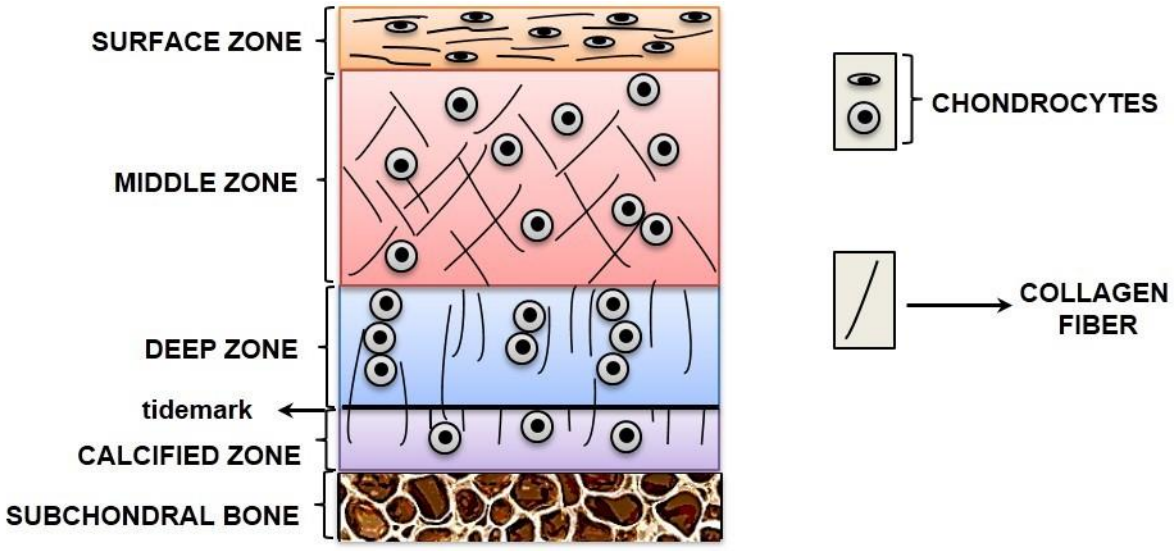


FIG. 1 Cartilage diacritical zones.

Chondrocytes

Although only *ca.* 1% of articular cartilage volume corresponds to chondrocytes, these cells are fundamental for tissue maintenance, as they substitute the molecules of the degraded matrix. Chondrocytes derive from mesenchymal stem cells (MSCs), which are present in adult bone marrow. During embryogenesis, MSCs differentiate into chondrocytes and continue dividing, going through several lineages, until finally becoming rounded, mature articular chondrocytes that cannot proliferate. [36,37]

The properties (*e.g.* degradation) of polymers commonly used in cell scaffolds for cartilage tissue engineering can be influenced by the type of cell used. Many studies on scaffolds built with chondrocytes have been reported. However, long periods of pre-implantation cell-culture lead to fibrous cartilage.[38] Interestingly, Caplan *et al.* suggested that certain subchondral defects in rabbits can produce cartilage; however, when undifferentiated MSCs were used, however only a complete subchondral plate was observed.[39]

Extracellular matrix

The ECM appears porous and is permeable. It mainly comprises water (60% to 80%), followed by collagen (10% to 20%) and other components (*e.g.* elastin, fibronectin and proteoglycans), which are responsible for nutrient transport, rigidity, adhesion, differentiation, function and cell migration.[40] The highly fibrous components (*e.g.* collagen and elastin) provide rigidity and tension to cells, whereas glycosaminoglycans modulate cell union and the activity of growth factors.[41] Owing to the balance between its constituent fluids and proteoglycans, the ECM promotes the mechanical properties of cartilage. The principal components of ECM are detailed below.

Water

The nature as if it were a mechanical engineer has been forced to use bearings or shock absorbers for its flagship product: the human being. And it has been so effective that it simply compacts its non-structural components there where they come into contact with one another to avoid wear and tear on the bone system, the main support and protection of the body.[42] For thet reason, the water flows in and out of cartilage, enabling it to structurally adapt to stress. There is more water at the ECM surface (80%) than at its lower depths (60%), and water molecules in the ECM can be free or bound to GAGs.[40] The high-water content in the ECM is maintained through interactions among and between water molecules and GAGs—chiefly, via repulsion between negatively-charged GAG chains, which promotes absorption of unbound water molecules; and, to a lesser extent, via hydrogen bonds.[43 44]

In short, the cartilage is located at the endings of the bones (epiphyses), beteewn them and the cycle of movement causes consecutive processes as infinite as the life of the human being in which the liquid is absorbed and desorbe from the articular cartilage lubricating the contact surface between the bones. This is the basis of the McCutchen model for contact surfaces loaded simultaneously and which has been known as weeping lubrication and could understanding as the balance between hydrodynamic ideal behavior and hydrostatic behavior due to self-pressure of the natural movement.[45]

Collagen

Most of the collagen cartilage is Type II collagen (90% to 95%), which gives it great tensile strength. Small amounts of other collagen types (I, V, VI, IX, X and XI) are also found in the ECM. Type VI collagens are produced in the early phases of osteoarthritis, whereas Type X collagens are produced only during endochondral ossification, which is normally associated with cartilage calcification. Collagens are comprised of at least 29 triple-helix polypeptide chains composed mainly of glycine, proline and hydroxyproline. This structure promotes traction and shear properties in hyaline cartilage and stabilizes the matrix.[46] The collagen fibrils form a network throughout the cartilaginous matrix, in which the diameter varies from the surface (20 nm) to the lower depths (70 nm to 120 nm). It is in this region that intramolecular and intermolecular cross-links form between the lysine residues in adjacent chains. [42,47,48]

Proteoglycans

Proteoglycans are subunits of glycosaminoglycans. They endow articular cartilage with resistance to compression (*i.e.* rigidity and elasticity).[49] These complex macromolecules are composed mainly of chondroitin sulfate, of which there are two subtypes: chondroitin 4-sulfate (*keratan sulfate*) and chondroitin 6-sulfate (*dermatan sulfate*). Keratan

sulfate is more abundant but its proportion decreases with age, while that of dermatan sulfate remains constant. Biglycan, decorin and fibromodulin are even less abundant. Glycosaminoglycans bind to a protein core to form aggrecans or other proteoglycans. These aggrecans are joined by proteins linked to hyaluronic acid to form aggregates of proteoglycans. Proteoglycans are responsible for cartilage porosity, have an average life of approximately 3 months and a high capacity for water retention.

Decorin and aggrecan are found in equimolar proportions in the articular cartilage attached to fibrils. Their relatively small size translates to a smaller diameter in fibrils, which are generally thinner at the surface.[50-52] Type VI collagen forms tetramers that bind to decorin, forming a branched network in the pericellular zone,[53,54] and is associated with hyaluronic acid (this is another GAG but is not sulfated, lacks a protein core and does not form proteoglycans, PG) in the network.[55,56] Both molecules (decorin and aggrecan) are more concentrated in pericellular sites[54-56] and these micro-filamentous structures can be found in that zone.[57]

Stockwell *et al.* has been studied the variation in the PG/GAG ratio of cartilage surface. For instance, they cut slices of cartilage adjacent to the articular surface, and then used tissue (Alcian Blue) staining to measure the distribution of GAGs and the levels of uronic acid and hexose. They found the highest levels of GAGs in the middle zone.[58] Franzen and colleagues performed a biochemical study to determine PG content in cartilage sections extracted from the deep zone.[59] They found chondroitin sulfate in the intermediate zone, and similar levels of non-aggregate PGs in the intermediate and the deep zones. However, immunohistochemical studies of PG variation in articular cartilage have not been enough. Poole *et al.* used immunofluorescence and sheep antibodies (S27) to bovine articular cartilage proteoglycan monomer and rabbit antibodies (R131) to bovine nasal cartilage link protein to study PG and the link proteins, finding them to be distributed all over the cartilage, with the highest concentrations in the perichondrocyte region in a separate study based on an immunoperoxidase technique and electron microscopy, the same group found variable distribution of PG and link proteins in function of the area of hyaline cartilage.[60]

Extracellular glycoproteins

The extracellular glycoproteins link chondrocytes to the ECM. Among the most important of these proteins is integrin, which regulates cell migration, proliferation and differentiation. Continuous renewal of ECM components depends on intracellular and extracellular proteases. Normally, cartilage exhibits high levels of protease inhibitors; accordingly, alterations in the levels of proteases and their inhibitors are a major cause of osteoarthritis.[41,61,62]

Three types of molecules that interact with chondrocytes are found in the tissue matrix: non-collagenous proteins, proteoglycans and collagens.[63] Type II, IX and XI collagens provide tensile strength and cartilage stiffness due to the fibrillar network they form. Type VI collagen helps chondrocytes to bind to the matrix macromolecular framework and is one of the matrix components that surrounds chondrocytes. The aggregating proteoglycans or aggrecans give to cartilage a longer duration, rigidity to the compression and elasticity. Small proteoglycans such as biglycan and decorin, provide stability to matrix through its union with other molecules. They can also bind to growth factors and regulate the chondrocytes function. One of the non-collagenous proteins called Anchoring CII helps chondrocytes anchor to cartilage's matrix.

Cartilage is remodeled internally and continuously, as chondrocytes replace the matrix macromolecules lost in the degradation process. Obviously, the remodeling of matrix depends on the capacity of chondrocytes to detect, and respond to, changes in their organization and in the composition of surrounding macromolecules (including by degradation), and to replace old molecules with new one. The matrix, in turn, acts as a signal transducer for chondrocytes.

Table 1. Articular cartilage components, percent, characteristics and functions

Cartilage components	Relative abundance (%)	Characteristics and functions
Collagen	10% (by total weight)	Type II collagen corresponds to roughly 90% to 95% of the dry weight of ECM and forms a network that is stabilized by the other types of collagen (I, IV, V, VI, IX and XI), which are found at much lower concentrations.
Proteoglycans	10% to 15% (by total weight)	Essential for normal cartilage function; examples include decorin and aggrecans.
Chondrocytes	2% (by volume)	Small, flat cells that are the predominant cell type in the ECM.
Water	70% to 80% (by total weight)	The principal component in hyaline cartilage; it transports nutrients to cells (chondrocytes) and lubricates tissue.
Extracellular matrix (ECM)	65% to 80% (by total weight)	Comprised mainly of water, collagen and proteoglycans and includes small amounts of glycoproteins, lipids, etc.

TISSUE ENGINEERING

Tissue engineering is a promising technique for cartilage regeneration. This approach encompasses four elements: cells, growth factors, scaffolds and mechanical properties (Figure 2). Following a summary of the principles of tissue engineering, advances in biomaterials used to engineer tissue structure and function will be reviewed. Focus is placed on biomaterials for tissue engineering, which are more biologically interactive and mimic some of the regulatory aspects of the ECM. Building upon the principles of tissue engineering and material design strategies presented, specific dental and craniofacial applications, including engineering of teeth, periodontium, skin, oral mucosa and salivary glands will be discussed with emphasis on application of cells, scaffolds and signalling strategies.

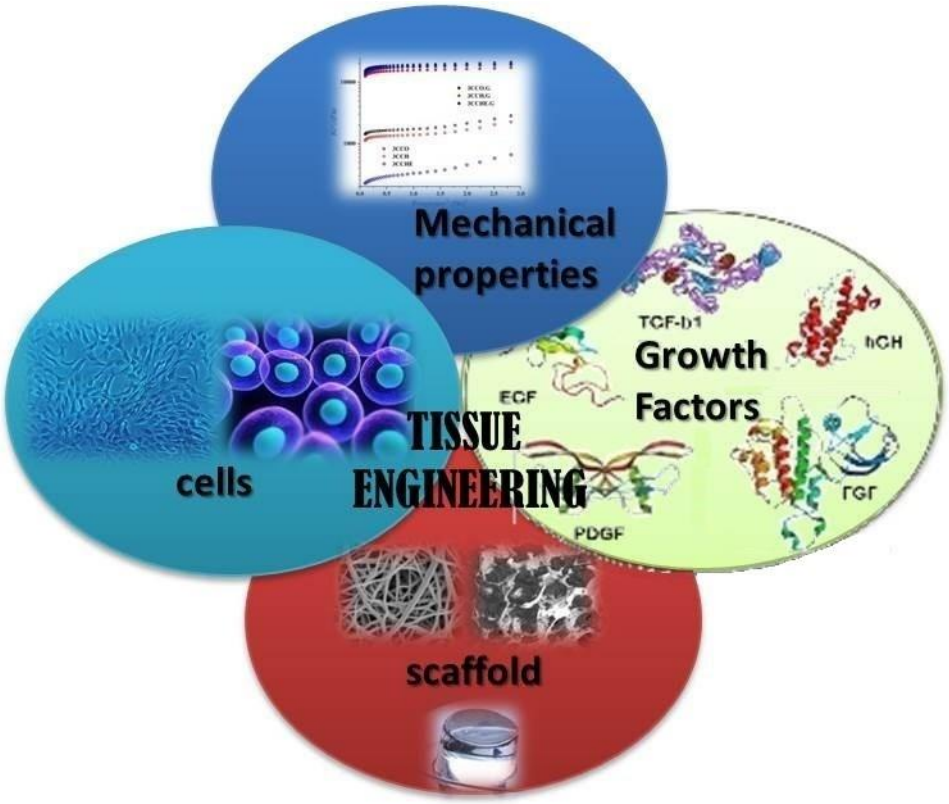


FIG. 2 The elements of tissue engineering for cartilage restoration.

Cells

The cells used in tissue engineering can be extracted directly from the cartilage (which yields chondrocytes) or obtained through chondrogenic differentiation of MSCs extracted from various mesenchymal tissue types (*e.g.* adipose, periosteum, bone marrow or synovia) and subsequently treated with growth factors. However, chondrogenic differentiation is highly complex, so in many studies of cartilage tissue engineering, researchers have preferred to use mature chondrocytes. Moreovr, when they proliferate they de differentiate.

Chondrocytes are an attractive choice for the seed cells to be used in scaffolds for cartilage tissue engineering because they produce both ECM and the subsequent type II collagen that formed it. These cells can be collected in healthy regions of articular cartilage where there is no mechanical load, and then cultured and expanded *in vitro*. However, this procedure has a major drawback: once chondrocytes have passed to a two-dimensional culture, they can lose their chondrogenic phenotype and differentiate into other cell types.[64] Efficient production of hyaline cartilage demands that chondrocytes maintain their phenotype and that they do not produce any type I collagen. Another important aspect is that as patients age, the aggrecans produced by their chondrocytes become smaller and less uniform aggrecans. Thus, older chondrocytes exhibit lower biosynthetic and mitotic activities, and weaker responses to growth factors.[65]

Cartilage can be integrated by inducing migration of free chondrocytes from the matrix to the tissue. For example, Pabbruwe and colleagues devised an implantable system based on a collagen scaffold with chondrocytes to distribute the cells between two surfaces of cartilage.[66] They isolated chondrocytes from bovine nasal septum and seeded on both surfaces of a collagen-based scaffold. They used a model of two discs of cartilaginous tissue, into which was interspersed the collagen/chondrocyte scaffold system to achieve *in vitro* integration. The researchers kept the scaffold in culture for 40 days, after which point the resulting tissue was analyzed histologically and biomechanically. They concluded that chondrocyte supply could be controlled, and that cartilage restored could be regenerated using a chondrocyte/collagen scaffold system. There have been several studies on the supply and distribution of cells for repairing a lesion in a joint area. Likewise, the characteristics of progenitor cells, and the mechanisms behind their chondrogenic differentiation, have also been investigated. For instance, Solchaga *et al.* have attempted to repair cartilage tissue through differentiation of MSCs at the damaged site.[67] They studied human MSCs, methods for isolation and expansion of these cells, and their qualitative and quantitative differentiation into chondrogenic cells.

Growth factors

Several growth factors are generally associated with maintenance of the chondrocyte phenotype and with chondrogenic differentiation of stem cells *in vitro*, including transforming growth factor β1 or 2 (TGF-β1 or TGF-β2), insulin-like growth factor (IGF-1), growth and factor differentiation 5 (GDF-5) and bone morphogenic proteins (BMP-2, BMP-4 and BMP-7).[68]

When chondrocytes are expanded in culture, treated with growth factors and seeded in a damage hyaline tissue, TGF-β1 drives largescale synthesis of DNA and glycosaminoglycans due to increased cartilage expression of type II collagen. Additionally, BMP 2 and BMP 4 stimulate the formation of cartilaginous tissue and GDF-5 triggers increased production of pre-chondrogenic precursors and the transcription factor sox-9.[64,69] Interestingly, in MSCs, BMP-4 stimulates chondrogenesis, thus promoting their differentiation into mature chondrocytes.[70] The growth factor IGF-1 has anabolic effects on chondrocytes, prompting them to synthesize ECM by favoring production of type II collagen and proteoglycans, preventing the release of proteoglycans and inhibiting their degradation. Interestingly, Fortier *et al.* evaluated the efficacy of IGF-1 for promoting ECM biosynthesis. After seeding mature chondrocytes either with or without IGF-1, they observed considerably higher levels of aggrecan mRNA and type II collagen in

the IGF-1-treated cells, which maintained their characteristic rounded phenotype. They also observed in the IGF-1-treated cells a dose-dependent increase in total collagen and GAGs, as well as lack of type I or IIA procollagen, which indicated that these cells had not undergone dedifferentiation.[71] Other studies by this group suggested that fibrin composites with IGF-1 grafted in extensive articular defects reinforces the bonds between damaged cartilaginous tissue and subchondral bone, while increasing the proportion of chondrocytes, as demonstrated in a greatly improved histological score.[72] After 4 weeks, the transduced chondrocytes already showed a 100-fold increase in type II collagen; at 8 weeks, there was already a greater amount of articular cartilage covering the lesion, and the histological scores were improved compared to the controls.[73] Van Beuningen *et al.* have investigated other growth factors and concluded that, *in vitro*, biosynthesis of proteoglycan chondrocytes is stimulated by molecules associated with BMP-2 and TGF-ß1 and that, *in vivo*, these factors also influence proteoglycan metabolism in hyaline cartilage.[74]

Researchers have explored the influence of type II collagen on the chondrogenic response of Extracellular Matrix. For example, Bosnakovski and co-workers studied bovine bone marrow mesenchymal stem cells MSCs cultured in different hydrogel-based based on type I collagen, type II collagen and alginate.[75] They treated the cells with media: a serum-free medium anda TGF-β1-supplemented medium. They observed chondrogenic differentiation in the type-II-collagen hydrogels after 72 hours of culture, and this differentiation increased with time, as verified by the presence of glycosaminoglycans and type II type I collagen the ECM. They also found that TGF-β1 had strongly facilitated chondrogenesis. As optimal conditions for expression of the chondrogenic phenotype, the researchers employed TGF-β1, dexamethasone and type II collagen. They concluded that type II collagen only induces and maintains the expression of MSC chondrogenicity, whereas TGF-β1 improves MSC differentiation.

Scaffolds

The scaffolds used in cartilage tissue engineering are based on carbohydrates (*e.g.* alginate, chitosan, poly-L-lactide /poly(glycolic acid) PLLA/PGA, agarose and hyaluronic acid), and proteins (*e.g.* collagen and gelatin). Growth factors are used to stimulate the development of the cells that will be implanted onto the scaffold and to maintain their chondrogenic phenotype.[76]

The main objective with engineered tissue is to mimic the structure and function of native tissue. Accordingly, a scaffold for cartilage repair must enable growth and proliferation of chondrocytes or MSCs, allow for free movement of these cells throughout its structure, and exhibit similar mechanical properties to native hyaline cartilage.

Furthermore, implantable devices must be biocompatible: thus, all scaffolds designed for clinical use, and their biodegradation products, must be innocuous to the host. Thus, when planning scaffold fabrication, researchers must consider any possible local effects on subjacent tissue, as the scaffold gradually breaks down and is replaced by host cells. Crucial factors to consider include the possible release of chemical cross-linking agents and effects of degradation byproducts on local pH levels. Moreover, tissue-repair scaffolds must be sufficiently porous be preloaded with cells and enable subsequent ingrowth of new tissue. Furthermore, they must be mechanically robust enough to withstand implantation as well as the mechanical loads typically placed on the joint surface. Lastly, once implanted, scaffolds must be able remain in place; indeed, if they are easily dislodged, then the cells or growth factors that they deliver locally will have minimal utility.

Ensuring that cartilage scaffolds persist long enough so that, over time, they become fully replaced by neo-cartilage is complicated, because osteochondral devices must fulfil the requirements of two different tissue types: bone and cartilage. Bone may tolerate a device over a longer period, but if subchondral bone is not regenerated quickly enough, the overlying cartilage will not be repaired correctly. In a review of matrices for cartilage repair, Coutts *et al.* emphasized that scaffolds should allow for cell attachment both to aid in retention of the implanted cells and to facilitate ingrowth by native cells.[77]

Mechanical properties

Mechanical properties such as compression, fluid-promoted shear stress and hydrostatic pressure must be considered when designing a system for joint-cartilage repair. Since tissue engineering aims to improve patients' quality of life, functionality of cartilage replacements is crucial—namely, to reduce the costs of, or delay, a possible joint arthroplasty or other interventions.

Mechanical stimulation is very important for the development of cartilage in infants and children,[78,79] as well as for *in vitro* chondrogenesis and tissue regeneration in adults, through the positive control of genes and the maturation of MSCs.[80,81] Efficient growth of neocartilage demands a specific combination of values for Young's Modulus, lubricant coefficient and viscoelasticity.[82-85] The similarity in mechanical properties between the graft material and the native tissue is cardinal for the functionality of the new cartilage at the macroscopic and microscopic levels. It is especially important in the repair of relatively large defects, because the mechanical loads must be supported effectively by the underlying tissue.

When the chondrogenic phenotype is maintained *in vitro*, cartilage can be regenerated by simply using an appropriate combination of scaffold and cells. However, to graft a scaffold *in vivo*, the implanted material must permanently bond to the local native tissue under natural joint conditions. Interestingly, subjecting the scaffold to biomechanical loads *before* implantation generates an adequate phenotype.

Walking causes articular cartilage to sustain myriad cyclic mechanical loads, including of hydrostatic pressure. Accordingly, chondrocytes must live under constant pressure. In a study on this phenomenon, Hu and Athanasiou produce tissue-engineered constructs over agarose *in vitro* and then subjected some of the cells to hydrostatic pressure (10 000 Pa) at 1Hz for 4 hours per day, 5 days per week, for 8 weeks. In lacunae, the cells that had withstood the pressure exhibited higher levels of collagen, a more-rounded phenotype and lower levels of GAGs than did the control (not subjected to pressure) cells. When simulating the mechanical environment in which chondrocytes should grow naturally, they are able to secrete growth factors and differentiate them by an appropriate route.[86].

SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING: COMPONENTS AND ARCHITECTURE

During the advent of tissue engineering, in the early 1990s, researchers explored different combinations of genes, cells, proteins, growth factors and porous scaffolds. Biomimetic scaffolds have emerged to try resembling articular cartilage in terms of structure, function, extracellular matrix and they are promising materials for restoration and / or repair of cartilage tissue. These biomaterials are designed to simulate natural environment of ECM, which provides diverse chemical, physical and biological signals for cell growth and function.[87,88] Thus, creating an optimal cellular microenvironment for proper growth of 3D cartilage requires a biomimetic scaffold that has readily tunable mechanical and physical properties, adheres strongly to cells and releases growth factors. Moreover, a scaffold for articular cartilage repair must be highly porous, with a pore size that facilitates cell adhesion, cell proliferation and ECM production, and connections between pores, to enable exchange of nutrients among cells.[89] Lastly, the constituent material must be biocompatible with cartilage; bioresorbable, with an adequate rate of degradation; and have mechanical properties and an architecture similar to those of cartilage, to promote the regeneration of native tissue and ensure a clinically useful size and shape.

Scaffold components

Attractive building blocks for tissue-engineering scaffolds include natural polymers, proteins, carbohydrates and hydrogels owing to their biocompatibility and biodegradability. Additionally, these compounds facilitate binding of implanted scaffolds to cartilage tissue *in situ*.

Hyaluronic acid

Hyaluronic acid is a fundamental component of cartilage tissue matrix. Cross-linked forms of hyaluronic acid have been studied as cartilage repair scaffolds. For example, Butnariu-Ephrat *et al.* implanted marrow MSCs in a hyaluronic acid-based glue in damaged goat cartilage, and then, shortly after surgery, observed that the repaired tissue appeared different than neighboring, normal articular cartilage.[90] Knudson and co-workers determined that hyaluronic-acid oligosaccharides induce chondrocytic chondrolysis, including total loss of stainable proteoglycan-rich matrix and activation of gelatinolytic activity.[91] Other groups have used scaffolds based on hyaluronic acid (either alone or combined with calcium phosphate), loaded them with MSCs and implanted them in rabbits for cartilage repair, achieving good results.[92-94] However, the cartilage formed using these implants appeared thinner than the host's normal cartilage. Solchaga *et al.* compared the outcome of osteochondral defects in rabbits filled with a fibronectin-coated hyaluronan-based sponge (ACP™) with or without autologous bone marrow. The fibronectin-coated hyaluronan-based scaffold organized the natural response and facilitate the integration of the neo-cartilage with the tissue.[94] Marcaci *et al.* employed a three-dimensional hyaluronic acid matrix to culture autologous chondrocytes, and then implanted this matrix in a human knee without the use of a periosteal flap, which enabled a reduction in transplant site morbidity by using arthroscopy as the surgical technique as compared to classic autologous implant.[95]

Solchaga *et al.* compared in another study the effect of two seeding cells methods (vacuum-aided seeding technique and passive infiltration) on the retention rate of hMSC in hyaluronic acid sponges.[96] In all *in vitro* tests the vacuum-aided seeding technique presented better results than the passive one. The objective of this research was to establish a simple and reproducible protocol for uniform seeding of cells in preformed porous scaffolds in large-scale (14-mm diameter by 6-mm thick).

Collagen

Type I collagen is a natural component of skeletal tissues such as bone tissue. Accordingly, collagen-based scaffolds have certain advantages over other types: for example, they allow for contact between the pre-loaded cells and endogenous cells located in joint tissues.[97,98]

Collagen-based biomaterials can be fabricated by enriching a collagen solution with biomolecules such as elastin,[99] chitosan[100-102] or GAGs.[103-105] The collagen required for scaffolds can be extracted from biological tissue by using acidic,[106,107] neutral-saline[108,109] or proteolytic[110-112] solutions. Proteolytic solutions are not highly recommended

because they alter the molecular structure of this biomolecule blocking terminal telopeptides, leading to an irremediable reduction in assembled tropocollagen fibrils.[113] Interestingly, endogenous proteases can be inhibited during solubilization of type I collagen acidic solution.[114] Regardless, the extraction technique that provides the highest yield is solubilization of type I collagen acidic solution containing pepsin, which leads to minimal blocking or denaturing of telopeptides.[114,115]

For decades, collagen-based scaffolds have been widely used for characterization of chondrocytes and stem cells *in vitro*[116] as well as *in vivo*, in rabbits,[97,117] sheep,[118] horses,[119] and dogs.[120] Collagen matrices that contain GAGs have been explored for gene therapy.[121] Encouraging results have been obtained using collagen-fiber scaffolds to deliver chondrocytes[97] or BMPs[117] in rabbits. For instance, Frenkel and co-workers reported that, 6 months post-implantation in rabbits, a collagen fiber-based scaffold loaded with chondrocytes induced articular-type repair similar to native tissue.[97] Analogously, Sellers *et al.*, described cell-free, BMP-2 loaded type I collagen implants that afforded excellent repair. They later assessed defects of clinically relevant size, applying appropriate means of implant retention, to confirm the clinical efficacy of repair.[117]

Deponti *et al.* assessed autologous chondrocytes for seeding of a collagen scaffold.[122] Initially, they isolated chondrocytes from infant hyaline cartilage, and then rapidly seeded these cells in collagen-based sponges immersed in medium. To optimize the seeding conditions, they evaluated the effects of adding fibrin glue on cell survival within the sponge and tested different time periods of *in vitro* scaffold maturation. They subsequently expanded the cells *in vitro*, re-suspended them in fibrinogen, seeded them into collagen scaffolds and finally, cultured them for 1, 3 or 5 weeks. They evaluated the different cultures to determine the optimal time for the rescue of chondrogenic phenotype, which they determined to be 3 weeks. Ultimately, they developed a collagen-fibrin adhesive sponge that could efficiently support cell survival and synthetic activity in culture, and they demonstrated the feasibility of converting modified specimens into tissue with chondral properties *in vitro*.

Chitosan

Another popular constituent material for biomimetic scaffolds is chitosan,[123] which is a partially deacetylated polymeric derivative of chitin, which is commonly found in the cell walls of fungi and in the shells of crustaceans.[124] Chitosan comprises a network of β(1-4)-linked glucosamine units that also contains N-acetyl-glucosamine units. The ratio of glucosamine units to N-acetyl-glucosamine units determines the degree of deacetylation in the polymer, which varies from 30% to 95%. The molecular weight of extracted chitosan ranges from 300 kD to 1000 kD,

depending on its source and the method used for preparation and purification. The solubility of crystalline chitosan in aqueous solutions is pH-dependent: above pH 7, it is practically insoluble, but from pH 6 downwards, it begins to become soluble, due to protonation of its free amino groups.[125-127]

The proteoglycans and GAGs in cartilage, which are anionic, partake in electrostatic interactions with chitosan, which is cationic. This phenomenon is extremely important for retention and concentration of growth factors at the implant site, as the growth factors are related to GAG (mostly with heparin) and it is very convenient that the scaffolding based on this polymer incorporates a chitosan complex.[127] Chitosan oligosaccharides also stimulate macrophages, both *in vitro* and *in vivo*.

For orthopedics, chitosan has been widely used in combination with materials such as calcium phosphates, hyaluronic acid, poly-L-lactic acid, alginate, polymethyl methacrylate and growth factors for bone and cartilage restoration and/or regenration. Chitosan is also frequently used in tissue engineering to work with cells.[128] Reported examples of chitosan-based scaffolds for cell culture included gels,[129] sponges and fibers,[130] porous materials based on a mixture of chitosan and ceramics,[131] and polymeric materials that contain gelatin or collagen I or II,[132,133] added to facilitate cell seeding and enhance the mechanical properties of the implant.

Jeon and co-workers compared a chitosan-based scaffold to a PLGA-based scaffold for *in vivo* cartilage repair in nude mice.[134] They seeded the scaffolds with cells, and then implanted them subcutaneously in the animals. The chitosan-based scaffold maintained its volume up to 12 weeks post-implantation, and had degraded upon formation of mature cartilage, at 16 weeks. In contrast, the PLGA-based scaffold material exhibited good cartilage development at 4 weeks post-implantation but was reabsorbed / absorbed by the 12th week. The authors postulated that the porosity of chitosan may have delayed the formation of new cartilage, but its longer life compared to PLGA enabled greater maturation of the ECM network.

Polylactic acid

Caterson *et al*. reported that marrow MSCs cultured in polylactic acid or polylactic acid/alginate scaffolds and treated with exogenous TGF-β1 undergo chondrocytic differentiation.[135] Along these lines, Frenkel and colleagues created, and tested in rabbits, a scaffold based on D,D-L,L-polylactic acid and collagen, which featured separate tissue-specific environments for the regeneration of cartilage and bone.[136] As with other polylactic acid scaffolds, its mechanical properties provided secure positioning at the implantation site, thus obviating additional fixation. The composite, treated with BMP-2, induced the growth of high-quality, articular-like tissue that remained up to 24 weeks

post-implantation and integrated well with host tissue. This group of researchers have performed similar studies in large animals.

Elastin

Elastin is a protein that affords strength and flexibility to the connective tissue in cartilage, ligaments, skin, *etc.* It comprises approximately 800 amino acid residues[137] arranged in hydrophobic domains and cross-linking domains. It is normally found in ear and nasal cartilage but it can found in other articular cartilages. This structure provides mechanical properties to connective tissue while enabling elastin to form reticulated, stable structures with surrounding molecules.[138] Human elastin sequences have been widely used in polymer synthesis for tissue engineering—namely for providing control over the functional properties of peptides and the mechanical properties of biomaterials. A class of elastin-derived polypeptides that has been widely used in tissue engineering is *elastin-like polypeptide (ELPs)*, which are encoded by the tropoelastin gene and comprise repeating units of the pentapeptide VPGVG.[137] They offer excellent control over protein functionality, as their molecular weight and amino-acid sequence can be controlled with high precision at a synthetic and genetic level. Moreover, ELPs are biodegradable, biocompatible and immunogenic, thus explaining their widespread use in tissue engineering.[139] Betre and co-workers added ELP to a matrix for use in cultivation of chondrocyte monolayers, which enabled longer periods of growth without the dedifferentiation inherent to other methods. They found that non-crosslinked ELP facilitated rapid accumulation and retention of matrix associated with chondrocytes. The ELP solution underwent transition and coacervation at 35°C, ultimately trapping primary chondrocytes within the matrix. Since the transition of ELP was reversible, the authors could recover the matrix and the cells (for subsequent seeding in monolayers) after 10 days of culture by simply stirring them gently at room temperature. Encouragingly, the chondrocytes retained their phenotype for up to 4 weeks, as corroborated by their accumulation of type II collagen and GAGs.[140] Later, Betre *et al.* also demonstrated the utility of ELP for the culture and chondrocytic differentiation of stem cells.[141]

Alginate

Alginate is a linear anionic natural polymer composed of repeating units of disaccharides—specifically, blocks of the homodimers (1→ 4)-α-L-guluronic acid (GG) and (1 → 4)-β-D-mannuronic acid (MM), and the heterodimer (1 → 4)-β-D-mannuronic acid - (1→ 4)-α-L-guluronic acid (MG).[142] It is naturally abundant in brown algae (25% to 45% by dry weight), from where it is extracted.[1,2] The quantity and quality of extracted alginate depend on the algae species, the type and age, and the extraction method. Given its natural abundance and biocompatibility, alginate is

commonly used for biomaterials such as scaffolds for cell seeding.[143,144] Purified alginates are widely used in the pharmaceutical industries as stabilizers in solution and dispersion of solid substances. In the biomedical field, they are also used for various purposes, such as: drugs controlled release,[145] encapsulation of cells,[146] scaffolds for tissue engineering, either in ligaments or tendons.[147,148] Alginate in solution has negative charges unless there are non-monovalent cations that promote its "natural criss-cross" due to contraction according to the famous model of the egg box. However, this is beneficial for the three-dimensional structures prepared for the cartilage with this material because higher density of negative charge promotes better and greater cell adhesion.[53,149,150] Li and Zhang studied the biocompatibility of chitosan-alginate scaffolds in studies on the morphology, proliferation and function of chondrocytic HTB-94 cells.[151] They reported that the scaffolds promoted cell proliferation and improved the expression of chondrocytes, and they concluded that the chitosan-alginate hybrid could be an alternative to chitosan as a scaffold material for cartilage engineering.[151].

PGA, PLA and PLGA

Some researchers have explored the interactions between chondrocytes and the poly-(α-hydroxy ester) polymers poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(lactic-co-glycolic) acid (PLGA),[39,98,152-154] all of which are FDA-approved. These polymers are all degraded by hydrolysis. Although PGA is more crystalline and less hydrophobic than PLA, it also degrades more quickly.[155] Sittinger *et al.* studied the interactions between either of these polymers and human chondrocytes at constant pH for 12 days, and concluded that PLA was less cytotoxic than PGA.[152] Chu and co-workers seeded perichondrial cells in PLA meshes, and then grafted the loaded scaffolds into rabbit femoral condyles.[156] They observed firm hyaline cartilage at 6 weeks post-grafting. In a similar study, Ma *et al.* seeded bovine chondrocytes on PGA scaffolds, and observed cartilaginous morphology after 12 weeks.[157] Moreover, the mechanical properties of the engineered tissue resembled those of native tissue.

Freed *et al.* arrived to conclusions that the growth of bovine chondrocytes in PLA matrix was twice as high as in the PGA in less than 2 months, however, after six months of study, cell population was almost similar in both matrices. The difference in first stages is due to the fact that PLA degradation occurs much slower, and for this reason, there was not so much space for proliferation.[158]

Sherwood and co-workers 3D-printed a PLGA/PLA-based heterogeneous chondral scaffold as a biomimetic of articular cartilage.[159] They characterized the structure for porosity, composition, architecture and mechanical properties, finding local variations. Thus, the upper scaffold, which comprised D-L-PLGA/L-PLA, was 90% porous

and featured macroscopic channels to promote homogeneous cell seeding. Using a porosity gradient and materials to avoid scaffold delamination, they assembled a transition region. The lower area (like the bone tissue), made of L-PLGA/TCP to promote growth of bone tissue and good mechanical properties, was 55% porous. The authors seeded chondrocytes in the upper scaffold area (like cartilage). Histologic and biochemical analyses revealed that cartilage tissue had formed at 6 weeks *in vitro* culture. According to the authors, the mechanical properties (tensile strength) of the bone portion of their osteochondral scaffold resembled those of cancellous human bone, thus highlighting the promise of such frameworks for clinical applications, including total arthroplasty.

Scaffold architecture

Scaffold architecture is critical for enabling strong attachment of cells to the biomaterial, favoring their proliferation and favoring good mechanical properties. For a given scaffold, the choice of architecture depends on the target implant site and the intended function in the body.[46] Porous scaffolds can be assembled via numerous techniques, including gas foaming, temperature-dependent phase separation, membrane lamination, fusion molding, solvent melting and fiber bonding. However, research on these techniques remain too focused on process optimization, rather than on biomaterial design.

Fibrous scaffolds

One class of scaffolds that has been explored for repair of damaged cartilaginous tissue is fibrous scaffolds. Li *et al.* electrospun and characterized a set of fibrous polymer scaffolds, based on biodegradable poly-(hydroxy esters), which, when prepared under optimal electrospinning conditions, they considered suitable for tissue engineering applications.[160] Similarly, McCullen and co-workers fabricated a tri-laminar fibrous scaffold from poly(ε-caprolactone) (PCL) by electrospinning, varying the conditions and thickness of the fibers.[161] Owing to their robust support for restoring the damage cartilage, promotion of cartilaginous tissue growth *in vitro*, and superior mechanical properties (compared those of homogeneous material), such laminar scaffolds appear highly promising.

Porous scaffolds

Porous scaffolds also have been explored in the context of tissue engineering. For instance, Wu and Ding studied the *in vitro* degradation of porous (87 ± 3 %) 3D scaffolds based on amorphous poly (D, L-lactide-co-glycolide), which they exposed to phosphate buffer solution at 37 °C.[162] The authors evaluated the extent of degradation based on four stages: quasi-stability, loss of force, loss of weight and, finally, fracture. They observed encouraging results for the

mechanical properties of the wet material. Liao and colleagues assembled a biodegradable porous scaffold from chondroitin sulfate methacrylate (CSMA)/polyethylene glycol methyl ether, ε–caprolactone acryloyl chloride (PECA)/graphene oxide (GO) for cartilage tissue engineering and studied its degradation rate, swelling ability, conductivity and mechanical properties, reporting good values for all parameters with pore size range of 100-200 μm.[163] They also tested their CMSA/PECA/GO scaffold *in vivo*, observing suitable tissue and affirming the utility of this scaffold. Lastly, Jonnalagadda *et al.* developed porous scaffolds based on poly(ε-caprolactone)/polyglycolic acid (PCL/PGA), which exhibited pore sizes between 20 and 120 μm with 99 % of porosity approximately for repair of articular cartilage.[164] It demonstrated similar mechanical properties to those of human articular cartilage, plus high levels of chondrocyte adhesion, proliferation and GAG secretion.

Porous multilayer scaffolds

An alternative to seeding cells on a monolayer scaffold is to use a multilayer graft that promotes a higher growth of cells and guides the simultaneous regeneration of bone, cartilage and a calcified cartilage intermediate[165]. Porous multilayer scaffolds are advantageous because they require a smaller population of chondrocytes than the traditional cell-based monolayer approach, resemble natural cartilage in their complexity, and enable mimicking of the mechanical properties of the natural tissue in each layer (Figures 3 and 4). The "calcified layer" could be filled with a calcium phosphate (hydroxyapatite, β-tricalcium phosphate, octacalmium phosphate, etc.) to promote the osteointegration and osteinduction of the bone tissue, which is close to subchondral bone.

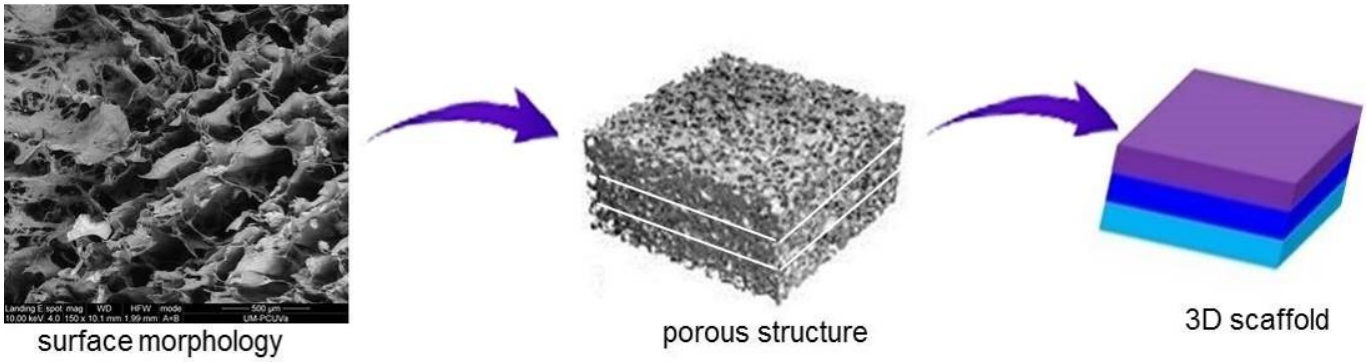


FIG. 3 Porous multilayer scaffold for regenerating cartilage tissue.

Camarero-Espinosa *et al.* recently designed and built a multilayer scaffold with nanometric features, in which they sought to mimic the mechanical properties, structure and chemical signals of hyaline cartilage.[166] Their scaffolds provided control of the orientation, morphology and phenotype of seeded chondrocytes. Furthermore, these scaffolds promoted the growth of new tissue that resembled articular cartilage, and favored the formation of localized apatite

nuclei, which facilitated integration of the scaffold into subchondral bone. Ng and colleagues devised a technique for creating agarose-based scaffolds by layering, reporting that such scaffolds demonstrated biomechanical properties suitable for chondrocyte seeding and potentially, for tissue engineering applications.[167]

Analogously, Wu *et al.* obtained collagen-based scaffolds via layer-by-layer casting and subsequent freeze-drying, generating a multilayer material with properties similar to hyaline cartilage.[168] The upper layer comprised collagen exclusively, whereas the lower layer was composed of collagen and hydroxyapatite. Between these two layers were intermediate layers, in which the collagen/hydroxyapatite ratio varied gradually. The authors cultured knee-joint chondrocytes from New Zealand rabbits in the upper layer, and after 2 weeks of culture, observed by immunohistochemistry and histology that the cells had maintained their phenotype. The general approach of a bilayer graft that guides the simultaneous regeneration of bone and cartilage has since been tested using various biomaterials and scaffolds,[169-182] and growth factors.[183-186] These studies underscore the need for a consistent biological barrier between the neocartilage and the bone region. However, bilayer design alone may not be sufficient to form structurally and functionally appropriate cartilage and bone, even when many studies support this structure.[97,172,184-186] Thus, trilayer scaffolds with cartilage, interface, and bone regions have been designed for osteochondral regeneration.[38,187-189]

For instance, Heymer *et al.* tested a trilayer scaffold (produced by Kensey Nash Corporation) that included a hydrophobic interface that separated the cartilage region (type I collagen fibers plus hyaluronic acid) from the bone region (type I collagen fibers plus PLA).[190] The authors seeded stem cells in the hyaluronic acid region above the interface and cultured them for 3 weeks, ultimately observing formation of cartilage-like tissue. Moreover, although calcified cartilage formation was not observed, the three layers remained distinguishable and structurally stable after culture. Alternatively, Jiang *et al.* reported a scaffold composed of an agarose hydrogel and bioactive PLGA/45S5 glass-composite microspheres, which supported the regiospecific co-culture of chondrocytes and osteoblasts. Their design enabled *in vitro* assembly of three compositionally distinct yet structurally continuous regions containing cartilage, calcified cartilage and bone-like matrices.[191]

Kon *et al.* developed an acellular trilayer osteochondral scaffold to control the spatial distribution of hydroxyapatite and collagen to facilitate the cartilage-to-bone transition. The upper layer comprised 100% type I collagen; the middle layer, 60% type I collagen and 40% hydroxyapatite; and the lower layer, 30% type I collagen and 70% hydroxyapatite.[188] The authors joined the layers via freeze-drying, and then tested them in an adult equine

osteochondral-defect model. At 6-months implantation, they observed distinct non-mineralized and mineralized regions, and the new tissue was very well integrated with the subjacent tissue, including cartilage and bone.[187]

Levingstone *et al.* investigated the structural and micro-architectural properties of a porous multilayer scaffold and the biological behavior of the material *in vitro*, determining the biocompatibility, the attachment and proliferation of cells on the scaffold and the ability of cells to infiltrate through the porous and distribute evenly throughout the construct, demonstrating the potenciality of this kind of biomaterial for osteochondral repair.[32]

Porous multilayer scaffolds: mechanical properties

During the scaffold design process the focus is oriented to the replication of the native healthy tissues complexity and mechanical properties. This complexity could be achieved by introducing in the scaffolds gradients of morphology, composition and function. Regular use of a joint subject to loads make cartilage to generate mechanical, physicochemical and electrical signals that allow chondrocytes to exert their synthetic and degradative activity and consequently, to remodel cartilaginous tissue. However, when a joint is not used regularly, the matrix changes, causing a loss in structural integrity and mechanical functionality. Furthermore, aging causes alterations in matrix composition and in chondrocytic activity that can significantly compromise the function of cells and tissue, leading to gradual degeneration of cartilage.[61]

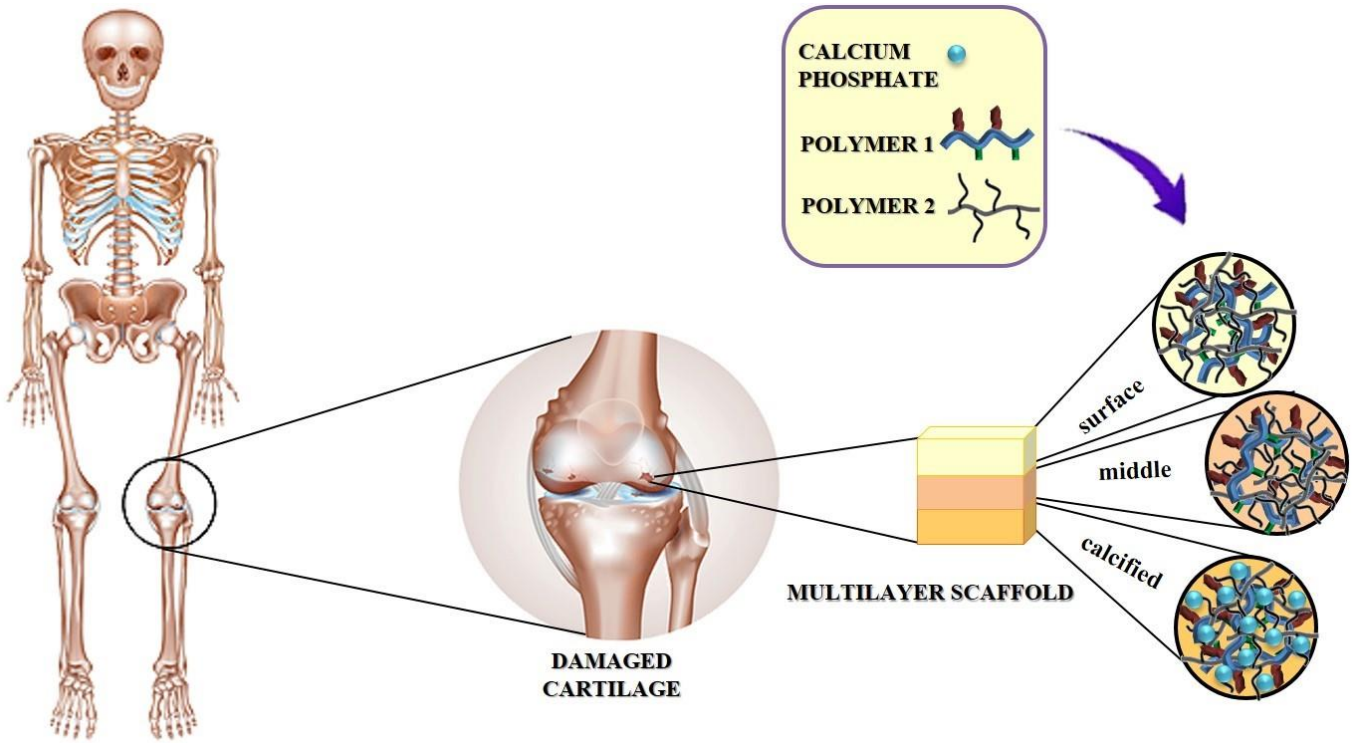


FIG. 4 Porous multilayer scaffold based on polymers and calcium phosphate for cartilage tissue engineering.

Collagen fibers[192-195] and scaffolds[196] have been characterized for mechanical function. Parameters that depend on specimen dimension are often described, including *round load* or *maximum load.*[192,195,196] These factors are important, as clinical applications may demand larger scaffolds than those evaluated in laboratory tests. There are also studies on intrinsic properties of materials, including *tangent module*[192,194-196] (which does not depend on the sample dimension) provides important information depending on the scaffold application. Other mechanical properties that must be assessed include *mechanical-load tolerance*, either *in vitro* or *in vivo*[197,198] and *viscoelasticity*, both of which influence the function of cartilage, ligament and other soft tissues.

The mechanical properties of non-traditional scaffolds have also been reported[63,68,199]. For instance, Schaefer and co-workers studied a tissue-engineering system based on chondrocytes that they had cultured in a bioreactor and subsequently seeded in a polyglycolic scaffold combined with an osteoconductive support.[200] They grafted this system into a large osteochondral defect in the femoropatellar groove in adult rabbits. Six months post-implant, the material supported physiological loads, had developed subchondral tissue and exhibited a Young's modulus like that of normal hyaline cartilage.

CONCLUSION

Researchers have designed diverse scaffolds for tissue-engineering applications with the long-term goal of clinical cartilage repair. However, despite their best efforts to mimic natural cartilage tissue, they have not yet reached its level of structural and functional complexity. Among the greatest achievements to date has been the development of porous scaffolds that enable internal and external cell adhesion and proliferation and that can withstand the mechanical loads applied to the damaged areas. Nevertheless, despite this and numerous other milestones, state-of-the-art materials continue to suffer from drawbacks—most importantly, once implanted, they fail over time, as demonstrated in animal studies. Thus, scientists are tasked with creating a new generation of scaffold biomaterials that are better suited for long-term cartilage regeneration *in vivo*. So far, multilayer cell-scaffold complexes based on natural polymers have had greater similarity in terms of composition and properties to cartilage and have achieved a appropriate restoration of the native tissue.

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Disclosure Statement

The authors declare no competing financial interest

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