# Biopolymer-Based Hydrogels for Cartilage Tissue Engineering

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# Biopolymer-Based Hydrogels for Cartilage Tissue Engineering

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## 1. INTRODUCTION

Replacement of body parts has been studied for at least 2500 years, when missing teeth were substituted with artificial teeth engraved from the bones of oxen.[1] Artificial substitutes are most often not as useful and long-lasting as the original tissues, and transplantation of real organs is severely limited by the shortage of donors.[2] Even when such organs are available, disease transmission and recipient rejection are the limiting factors.[3] With the advent of technological advances in cellular and molecular biology, engineering, and materials science, tissue engineering is budding to fulfill this long sought dream. Tissue engineering has emerged from the use of biomaterials which replace small area of damaged tissues to three-dimensional matrices which can act as cell carriers as well as signal providers for regeneration. Cartilage regeneration is an important target for tissue engineering as the incidence rate of arthritic diseases due to aging and obesity is very high[4] and, therefore, has a major impact on the quality of life. The concept of regenerating cartilage using scaffolds that can be either implanted or injected locally at the injury site can provide promising treatment approaches for articular cartilage defects.

Biopolymers are polymers which are of natural origin and form hydrogels which mimic the extracellular matrix (ECM) and promote tissue growth. Many biopolymers are in fact components of the ECM and can regulate division, adhesion, differentiation, and migration of cells more favorably than synthetic polymers.[5] There are several reports that suggest the beneficial cellular responses of biopolymers and their performance over synthetic polymers for cartilage tissue regeneration.[6−9] For example, Klein et al.[6] showed that alginate gel out performed poly(ethylene glycol) (PEG) gels in terms of expression of collagen type II and aggrecan and GAG deposition. Similarly, addition of chondroitin sulfate C enhanced mRNA expression of collagen type II of porcine chondrocytes cultured on poly(lactic-*co*-glycolic) acid mesh.[7] Compared to synthetic polymers, biopolymers have more functional groups and thus offer diverse and highly selective coupling chemistries.[10] The present review focuses on biopolymer-based hydrogel scaffolds for cartilage tissue engineering.

Cartilage development within a tissue-engineered construct depends on the structural environment, cell−biomaterial

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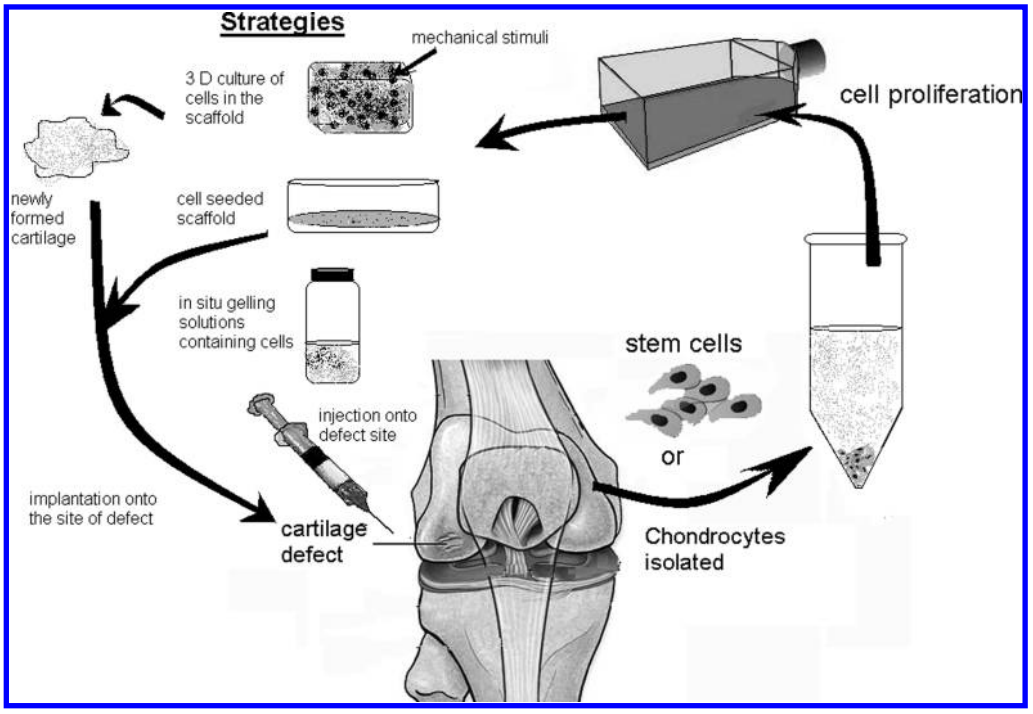


**Figure 1.** Schematic representation of methods of cartilage tissue engineering.

interactions, biological signals, mechanical stimuli, pH, and osmolality. This review also discusses the effects of cell sources, modification of physical parameters of scaffolds, and signaling agents on the cartilage regenerative capacity. While tissue-engineered constructs based on biopolymers such as hyaluronate, chitosan, agarose, alginate, fibrin, collagen, and silk proteins have shown promising results with regard to chondrocyte regeneration, use of chondroitin sulfate hydrogels as such has had limited success which will be discussed in the following section (see section 2.1.2).

Minimal invasiveness and targeted delivery at the preferred site are the key advantages of injectable scaffolds.[11] Among the different biopolymer-based hydrogels, those made of alginate and fibrin are most extensively explored as injectable systems. In situ gelling systems are categorized in terms of the cross-linking methods in this review. The poor mechanical strength and fast degradation profile of biopolymer-based hydrogels limit their extensive usage.[12] Hybrid hydrogels prepared by combination of biopolymers as well as synthetic polymers explore the beneficial aspects of both in terms of biocompatibility, controlled degradation, and mechanical strength. Requirements of cartilage tissue-engineering scaffolds on clinical perspective are also discussed.

The growing interest in the development of scaffolds which satisfies all the critical parameters for tissue regeneration and increasing research programs dedicated to cartilage regeneration substantiate the need for such a review. Even though there are few books and journal articles dealing with various features of tissue engineering, this is first document focusing on biopolymer-based hydrogels for cartilage regeneration and various parameters to be watched and evaluated to achieve the goal.

### 1.1. Why Tissue Engineering Is Preferred over Conventional Methods for Articular Cartilage Regeneration

Articular cartilage is a complex, living tissue that lines the bony surface of joints. The main function of articular cartilage is to provide a low-friction surface that enables the joint to bear weight during different ranges of motion needed to carry out daily as well as athletic activities. However, articular cartilage has little or no capacity to repair itself, and any damage to it often requires surgical intervention. This may be due to the limited proliferating potential of chondrocytes and their catabolic response to pathological mediators and avascular nature that prevents immigration of regenerative cells except at conditions when the lesion provides access to marrow. The existing techniques like microfracture, mosaicplasty, autologous chondrocyte transplantation, and osteochondral allograft transplantation for treatment of cartilage defects have successfully relieved pain and improved joint function, but cartilage produced by these methods are often composed of collagen type 1 which is inferior to hyaline cartilage both chemically and mechanically.[13]

In the early 1970s, Dr. W. T. Green conducted a number of experiments to generate new cartilage using chondrocytes seeded onto bony spicules and implanted in nude mice. Even though the experiments were unsuccessful, he correctly envisaged that with the advent of innovative biocompatible materials it would be possible to generate new tissue by seeding viable cells onto appropriately configured scaffolds.[14] Clinical application of such an approach was first performed by Brittberg et al.[15] There are now a number of successful approaches to tissue engineer cartilage (Figure 1), including injection of cells into the lesion with or without scaffold implantation allowing regeneration to occur in vivo and implantation of natural and synthetic biomaterial scaffolds carrying allogenic/autologous sources of mature chondrocytes or chondroprogenitor cells and chondroinductive growth factors and subjected to suitable mechanical stimuli.[16−18]

The basic principle of cartilage tissue engineering involves isolation of cells that may be expanded in vitro and then either encapsulated in a three-dimensional matrix for proliferation or mixed with in situ gelling systems and subsequently implanted/

**Table 1. Methods of Hydrogel Preparation**

| biopolymers | method of hydrogel formation | refs |
|---|---|---|
| hyaluronic acid | esterification with benzoic acid and photo-cross-linking of its acrylic derivatives | 58−61,174,175 |
| chondroitin sulfate | photo-cross-linking of its acrylic derivatives | 179 |
| | polyelectrolytic complex formation with COOH group containing biopolymers | 83 |
| chitosan | physical gelation by addition of alcohol/ exposure of ammonia vapors | 85 |
| alginate | cross-linking with divalent metal ions | 87−94 |
| agarose | solution forms gel on cooling | 104−106 |
| fibrin | thrombin cleaves fibrinogen and polymerizes to form fibrin network | 115,123,124 |
| collagen | polymerize into a stable gel at neutral pH | 129−138 |
| silk | sol−gel transition in the presence of acid and ions | 147−150 |

injected into the site of injury (Figure 1). Cells may be procured by culturing primary autologous/heterologous chondrocytes,[15] mesenchymal stem cells,[19] and embryonic stem cells.[20] Scaffolds preserve cell infiltration, proliferation, and subsequent differentiation in response to signaling molecules and mechanical stimulation and provide initial mechanical strength. Chondrocytes are highly environment specific, and it has been found that the interaction of integrin receptors with chondrocytes results in the production of extracellular molecules such as type II collagen and aggrecan.[21] Growth factors such as platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor-2 (FGF-2), insulin-like growth factor (IGF-1), transforming growth factor-$\beta$1 (TGF-$\beta$1), and bone morphogenic protein-7 (BMP-7) and hormones like dexamethasone are shown to induce various signaling pathways that aid in transducing phenotypic expressions by chondrocytes.[22−25]

### 1.2. Critical Factors in the Design of Cartilage Tissue-Engineering Constructs

The challenge for cartilage tissue engineering is the growth of cells within the scaffold, producing new cartilage tissue with appropriate structure and properties, thus providing a natural repair that can later become integrated with the patient's tissues. Ideal properties of a cartilage tissue-engineering scaffold include biocompatibility, promotion of cell differentiation and neocartilage formation, biodegradability in response to neocartilage formation, porous structure, mechanical stability, adherence and integration with the surrounding native cartilage, and proper filling of the defect site. The critical factors involved in the design of the tissue-engineering construct are discussed below.

**1.2.1. Choice of Hydrogel Based on Cellular Response and Degradability.** Hydrogels are three-dimensional networks of hydrophilic polymers that can absorb water from 10% to 20% to about thousands of times their dry weight without undergoing dissolution of the polymer because of their cross-linked structure. The porous structure of hydrogels along with the presence of water allows the transport of low molecular weight solutes and nutrients into the hydrogel as well as the cellular waste out of the hydrogel, which are critical to cellular viability. Cross-linking methods can vary; they may be ionic, chemical, enzymatic, or photo-cross-linking (Table 1).[26] An ideal cell carrier material should be one that mostly mimics the naturally occurring environment in the articular cartilage matrix. Hydrogels based on biopolymers mimic many features of the extracellular matrix and thus have the potential to direct the migration, growth, and organization of cells during tissue regeneration. Many of these hydrogels demonstrate adequate biocompatibility and biodegradability which also make them an appropriate candidate for scaffold development. Biopolymers used for hydrogel−scaffold development for cartilage tissue engineering include polysaccharides such as hyaluronate, chondroitin sulfate chitosan, alginate, and agarose and proteins such as silk fibroin, fibrin, and collagen.

**Table 2. Desired Biochemical Properties of Cartilage Tissue-Engineering Constructs**

| |
|---|
| maintenance of chondrocyte morphology, viability, and proliferation |
| promoting chondrocyte differentiation |
| preserving the cell phenotype, upregulating the expression of |
| a. collagen type II |
| b. aggregan |
| c. the transcription factor Sox 9 |
| downregulating the expression of collagen type I |
| promoting the deposition of GAG |
| integrating with host tissue |
| biodegrading as the neocartilage formation proceeds |

**1.2.2. Cell Sources.** Different sources of cells have been explored for cartilage tissue engineering which includes chondrocytes, fibroblasts, stems cells, and genetically modified cells.[27] In cartilage, chondrocytes are limited in number and has to be expanded prior to use, which often causes dedifferentiation of cells. Design of scaffolds and selection of cells and growth factors should be in such a way to preserve the cell phenotype, upregulating the expression of collagen type II, aggregan, the transcription factor Sox 9, while downregulating the expression of collagen type I (Table 2). Even though there are other potential autologous sources for chondrocytes (auricular, nasoseptal and costal cartilage), each has different functions, structure, and composition; hence, the ultimate choice of chondrocyte is governed by the end application. Reports show that fetal and young chondrocytes have faster growth rates, rapid in vitro expansion, and greater chondrogenic potential (increased Sox 9 and type II collagen expression) over chondrocytes from older donors.[28] Factors that affect cartilage regeneration are summarized in Table 3. Initial cell seeding density and culture period also affect the composition, structure, and function of certain tissue-engineered constructs. Long culture duration with higher initial cell density can increase the biosynthesis by chondrocytes.[29]

**1.2.3. Mechanical Strength of Tissue-Engineered Construct.** The tissue-engineered construct should be able to maintain physiologic loads within the joints over a long term. For smaller articular defects the surrounding normal tissue can protect it from excessive loading, whereas in larger defects construct itself will have to support stresses within normal physiological levels (2−6 MPa or higher). Having a dynamic compressive modulus of 12−20 MPa, native articular cartilage can sustain such stresses. Hence, evaluation of the dynamic modulus of the engineered construct in unconfined compression

Table 3. Factors Effecting Cartilage Regeneration

| parameters | desired value | ref |
|---|---|---|
| initial cell seeding density | higher initial cell density (64 million cells/ml) | 29 |
| mechanical loading (dynamic compression)[a] | 2−10% strain or 0.5−1.0 MPa at physiological frequency 0.01 to 1.0 Hz | 31−33 |
| osmolality | physiological osmolality | 39 |
| extracellular pH | 7.2 | 40 |
| pore size[b] | 70−120 $\mu$m | 41 |
| growth factors | PDGF, TGF-$\beta$, FGF, BMP, IGF | 21−25,27 |

[a] varies with experimental set up. [b] depends on the material.

has to be performed to check whether the construct can sustain physiological loading environment.[30]

**1.2.4. Mechanical Stimuli.** Mechanical loading (dynamic compression, fluid shear, tissue shear, and hydrostatic pressure) has been found to have beneficial effects on chondrogenic differentiation and functionality. It has been reported that dynamic compression at moderate levels (2−10% strain or 0.5−1.0 MPa) and physiological frequencies (0.01−1.0 Hz) can promote the biosynthesis of collagen, proteoglycan (PG), and fibronectin[31−33] (Table 3). However, it is suggested that the response to mechanical stimuli may differ with scaffold material. Effect of dynamic compression of chondrocyte-seeded fibrin gels on matrix accumulation and stiffness studied by Hunter et al. showed that cyclic unconfined compression produced engineered tissue with less collagen and PG.[34] The compressed gels were softer possibly due to reduced extracellular matrix (ECM) accumulation and increased matrix catabolism indicating the inhibitory effect of oscillatory compression on the formation of healthy tissue-engineered cartilage in fibrin scaffolds unlike in agarose scaffolds. The authors attribute the contradictory results to the difference in the experimental set up used in both cases. In the case of agarose scaffolds, Mauck et al.[35] used intermittent periods of oscillatory compression (3 consecutive 1 h-on-1 h-off cycles per day) while Hunter et al.[34] used continuous oscillatory compression (24 h per day). The factors that influence the outcome of mechanical stimulation are the stage of differentiation, region of origin (superficial or deep) of the chondrocytes, and interaction of mechanical stimuli with oxygen tension and growth factors.[36−38]

**1.2.5. Osmolality and pH.** Another aspect of chondrocytes that needs to be taken care of is its osmotic sensitivity. Maximum PG and collagen synthesis was observed near physiological osmolality, while above and below the physiological range decreased synthesis was observed. The degree of osmolality depends on the amount of free ions in the medium solution, and therefore, exploring different levels of culture medium osmolality may provide insight into osmolality effects on chondrocyte biosynthesis.[39] The extracellular pH also influences the synthesis of matrix macromolecules by chondrocytes, and maximum GAG synthesis was observed at pH 7.2.[40]

**1.2.6. Pore Size.** Pore size is yet another important parameter that determines the penetration of cells in the scaffold. There are few arguments supporting scaffolds with smaller pore size as pores naturally present in normal cartilage matrix have a size of 2.5−6.5 nm, and pores smaller than 50 $\mu$m are generally recommended to improve the biomechanical strength of engineered constructs. However, studies by Griffon et al.[41] support the usage of large interconnective pores (70−120 $\mu$m) which can improve the cellularity and matrix content on dynamic culture using rotating wall vessel. They found that cell migration and tissue in-growth are less with scaffolds having a small pore size, which is a major constraint in the clinical application of those structures. Lien et al.[42] showed that in gelatin-based scaffolds with larger pore size (250 −500 $\mu$m) cells stopped proliferating and instead secreted more ECM, whereas the scaffolds with smaller pore size (50−200 $\mu$m) showed more dedifferentiated cells. The authors also reported that scaffolds made by different materials will have different preferred pore size ranges even for the same source of chondrocytes, and the material and its microstructure also influence differently the behavior of chondrocytes.

**1.2.7. Signaling Molecules.** There are certain signaling molecules such as growth factors which can accelerate and/or enhance cartilage formation which can be added to the culture medium for in vitro culture. It can also be incorporated within the scaffold as such or within certain carriers for in vivo delivery to achieve cellular differentiation and tissue formation. Growth factors accelerate the cartilage repair process by different mechanisms including recruitment of chondrogenic cells (chemotaxis), stimulation of chondrogenic cell proliferation (mitogenesis), and enhancement of cartilage matrix synthesis. Growth factors like TGF-$\beta$, PDGF-BB, FGF, BMP, and IGF along with bioactive molecules like hyaluronate, chondroitin sulfate, and insulin have been investigated either alone or in combination for their effects on cartilage tissue engineering.[22−25,27] IGF has an important role in homeostasis of cartilage, balance of proteoglycan synthesis, and breakdown, and IGF (25 $\mu$g/defect) incorporated with fibrin clot when placed in an equine full thickness cartilage defect (15 mm diameter) improved the quality and quantity of repair tissue and reduced synovial inflammation. Enhanced proliferation of chondrocytes was observed with concentrations of PDGF-BB from 4.7 to 300 ng/mL with a maximum number of cells at 75 ng/mL.[22] Although TGF can stimulate both osteoarthritic and normal human articular chondrocytes, its use for cartilage repair is limited as it also stimulates osteophyte formation.[43] The effect of combination of IGF-1 with BMP-7 (both 100 ng/mL) has been studied on both normal and osteoarthritic chondrocytes and found that the PG amount produced was greatest in the combination group.[44] Among fibroblast growth factors, FGF-1, -2, and -18 stimulated in vitro proliferation of chondrocytes more effectively than FGF-4 and -9 whereas FGF-10 showed a suppressive effect. FGF-2 required 0.5% serum for its proliferative effect to be seen. Synergistic effects were obtained when FGF-2 (0.06 nM) was used with insulin (1.7 $\mu$M), IGF-1 (13 nM), and TGF-$\beta$2 (0.04 nM), but with dexamethasone (1 $\mu$M) the effect was antagonistic.[45]

## 2. CARTILAGE REGENERATION USING BIOPOLYMER-BASED HYDROGELS

### 2.1. Polysaccharide-Based Hydrogels

Recently, the potential of polysaccharides as biomaterials has been widely recognized. Factors that contribute to this success of polysaccharide-based biomaterials include the following:

(a) the critical role of saccharide units in cell signaling,
(b) development of powerful new synthetic techniques with potential of automated synthesis of biologically active oligosaccharides,

(c) rapid expansion in tissue-engineering research and associated need for new materials with specific, controllable biological activity and biodegradability.[46]

Additionally, polysaccharides can easily form hydrogels by hydrogen bonding, ionic interactions, or chemical cross-linking. Here different polysaccharide-based scaffolds for cartilage tissue engineering have been overviewed.

**2.1.1. Hyaluronic Acid-Based Hydrogels.** Hyaluronic acid (HA) is one of the important GAGs present in cartilage and forms the major component of synovial fluid. HA has D-glucuronic acid and D-N-acetylglucosamine units linked together via alternating $\beta$-1,4 and $\beta$-1,3 glycosidic bonds (Figure 2). The advantage of HA in cartilage tissue engineering includes its inhibitory effect on fibronectic fragment-mediated chondrocytic chondrolysis,[47] anti-inflammatory effects,[48−50] inhibitory effects on prostaglandin synthesis,[51,52] proteoglycan release,[53] and degradation.[54] In addition, HA present in native cartilage mostly as an integral part of proteoglycans together with other GAGs and plays an essential role in the formation of proteoglycan aggregates.[55] Moreover, it is degradable by the enzyme, hyaluronidase, and free radicals. Therefore, hyaluronan would be an ideal matrix for cartilage tissue engineering. Several studies have incorporated HA in hydrogels to exploit its beneficial aspects in cartilage tissue engineering.[56,57] The incorporation of HA to collagen gels created an environment which was more conducive for chondrocyte proliferation and ECM production.[56]

Moreover, in order to achieve the desired structural organization and physiochemical characteristics, it has been esterified and used as a carrier for chondrocytes or bone marrow-derived mesenchymal stormal cells in the treatment of cartilage defects of the knee. A high extent of esterification of HA with benzoic acid results in water-insoluble benzyl ester (HYAFF 11), which has been used as a scaffold for the culture of human nasoseptal chondrocytes,[58] adult human and bovine hyaline chondrocytes,[59] and mesenchymal stem cells.[60] Esterified HA was able to maintain chondrocyte phenotype even after a long period of in vitro conditions downregulating the expression of some catabolic molecules, thus providing a good environment to support the chondrogenic differentiation. Hyalograft C is a tissue-engineered graft where autologous chondrocytes are grown on HYAFF 11 scaffold and has been evaluated clinically. Nehrer et al.[61] explored the midterm efficacy and safety of Hyalograft C grafts in a group of 36 patients with symptomatic chronic defects of the joint surface and undergoing surgery. Evaluation methods adopted were Lysholm score, which assesses the stability and pain in the knee joint, and International Knee Documentation Committee (IKDC), which assesses the functionality that includes disability and recovery of patients and found good to excellent results for 68%, 74%, and 87% of patients at 1, 2, and 3 years, respectively. Patients with single defects and under 30 years of age showed significantly better outcome in both evaluation methods, suggesting that the scaffold is a safe and an effective therapeutic option for treatment of articular cartilage lesions.[61]

HA has free OH, COOH, and *N*-acetyl groups which are the major targets for its chemical modification. Bulpitt and Aeschlimann[62] developed a method for cross-linking high molecular HA for the preparation of biocompatible and biodegradable hydrogels under physiological condition by introducing ester groups onto HA and subsequent substitution with a side chain containing an amino or aldehyde moiety. Low molecular weight HA was not used as it was expected to evoke inflammatory

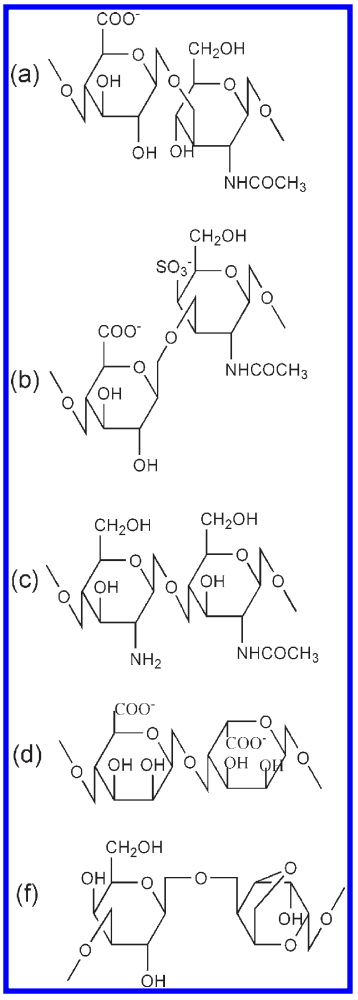


**Figure 2.** Structure of polysaccharides: Hyaluronic acid (a), chondroitin sulfate (b), chitosan (c), alginate (d), and agarose (e).

responses. Implantation studies of growth factors incorporated hydrogels showed a synergistic action of IGF-1 with BMP-2, which promoted cartilage formation, while addition of TGF-$\beta$3 and BMP-2 led to rapid replacement of the matrix by bone. An important issue that has to be addressed for cartilage tissue-engineering scaffolds is mechanical strength, and the poor mechanical properties of HA hydrogel are a disadvantage. However, by hybridization with various synthetic polymers like poly(*N*-isopropyl acrylamide) and poly(ethylene glycol), for example, an improved mechanical strength has been achieved as discussed in section 3 (Hybrid Hydrogels). Though preliminary evaluation of most of these hydrogels is promising, there are concerns over its availability, variability of the material properties depending on the source, and quality in terms of possible pathogen contamination.

**2.1.2. Chondrotin Sulfate-Based Hydrogels.** Chondroitin sulfate (CS) is a sulfated GAG composed of a chain of alternating sugars (*N*-acetylgalactosamine and glucuronic acid) (Figure 2). There are several reports to show its beneficiary aspects in preventing the prevalence of osteoarthritis. CS has been known to stimulate the metabolic response of the tissue both in vitro[63,64] and in vivo,[65] prevent the PG degradation in the osteoarthritis model in rabbit and, have anti-inflammatory properties.[66] Besides this, CS is highly negatively charged and can be enzymatically degraded by cellular secretion of chondroitinase.

The presence of CS has enhanced the expression of collagen type II mRNA in 3D culture of chondrocytes in a study by Nishimoto et al.[7] However, there appears to be contradictory findings in the literature regarding the utility of CS as a scaffold for cartilage tissue engineering. For example, Handley et al.[67] found inhibited collagen production in the presence of exogenous PGs in suspended cultures of aggregated chondrocytes due to a decrease in the synthesis of the collagen polypeptide chain, a precursor of procollagen. The presence of exogenous CS in the hydrogel may thus inhibit endogenous secretion of GAGs and collagen. It has been found that there is a progressive loss of endogenous GAG in long-term culture of explants in CS at 100 mg/mL, even while the chondrocytes remain alive and capable of maintaining the collagen content over time.[68] The authors interpreted this as the consequence of a feedback mechanism that downregulates GAG synthesis in response to the concentration of exogenous GAG present in the extracellular matrix.

Hydrogel scaffolds comprising of CS alone prepared by photopolymerization of its methacrylate derivative were also shown to inhibit chondrocyte biosynthetic activity.[69] The high concentration of fixed negative charge in the CS gels may attract free cations from the medium, resulting in an increase in osmolality within the hydrogel, making CS-based hydrogel not very successful in cartilage tissue engineering. However, CS hydrogel has been explored as an adhesive agent between scaffold and cartilage defect surface to improve integration with host tissue tissue.[70] Nevertheless, chondrocytes cultured in monolayers on chondroitin sulfate-modified chitosan membranes maintained their phenotype, increased type II collagen synthesis, and did not alter GAG production compared to those on polystyrene controls.[71] In another study where chondrocytes were seeded onto type I collagen scaffolds with covalently linked CS, enhanced cell proliferation and total PG production were observed but collagen synthesis was not addressed in the study.[72] It has been reported that PEG hydrogels containing CS can enhance chondrogenic gene expressions and cartilage matrix production.[73] Therefore, it can be assumed that though CS has beneficial aspects on cartilage tissue engineering while used along with synthetic polymers/biopolymers, use of CS hydrogel alone is skeptical. High cost also limits wide applicability of CS-based hydrogel scaffolds for cartilage tissue engineering.

**2.1.3. Chitosan-Based Hydrogels.** Chitosan is partially deacetylated chitin consisting of $\beta$ (1→4)-linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups (Figure 2). The *N*-acetyl-glucosamine groups in chitosan are also found in various GAGs present in articular cartilage, which have specific interactions with many growth factors, adhesion proteins, and receptors. Cationic nature and high charge density in solution help chitosan to form insoluble ionic complexes or polyelectrolyte complexes with a wide variety of water-soluble anionic polymers. One advantage of chitosan is that its physicochemical and biological properties can be drastically tailored by utilizing the reactivity of glucosamine residues which include acylation,[74] alkylation,[75,76] carboxymethylation,[77] quaternarization,[78] and grafting of chitosan with methacrylic acid and lactic acid.[79] The excellent ability of chitosan to process into porous structures is another advantage that has great applicability in cell transplantation and tissue regeneration. Porous structures are generated by freezing and lyophilizing chitosan solutions. Mechanical properties of chitosan scaffolds depend on the pore size and orientation. The porous membranes had greatly reduced elastic moduli (0.1−0.5 MPa) compared to nonporous chitosan membranes (5−7 MPa), and the highest extensibility was found with chitosan structures having a random pore orientation frozen rapidly at −78 °C to give pores 120 $\mu$m in diameter.[80]

It has been reported that injection of chitosan solution into the knee joint of rats caused a significant increase in the density of newly formed chondrocytes, suggesting that it could facilitate the wound healing of articular cartilage.[81] Further, derivative of chitosan, dicarboxymethyl chitosan, along with BMP-7 has induced the repair of cartilage lesions.[82] Chondrocytes attached to the polyelectrolytic complexes based on chondroitin sulfate and chitosan maintained round or polygonal morphology and had undergone only a modest degree of mitosis.[46] Chitosan has also shown a protective effect against GAGs hydrolysis by their specific enzymes when associated with polyelectrolytic GAGs.[83] Even though some inhibitory effects were shown by a lactose derivative prepared by Donatia et al.[84] on growth of pig articular chondrocytes in short-term cultures (up to 8−24 days), there was no evidence of any cytotoxic effects or inhibition of the GAGs and collagen biosynthesis. The derivative also stimulated the biosynthesis of chondro-specific aggrecan and type II collagen. Further, the intra-articular injection of chitosan has shown an increase in epiphyseal cartilage in the tibial and femoral joints with an activation of chondrocyte proliferation. Physical hydrogels prepared by addition of alcohol/exposure of ammonia vapors to chitosan in acetic acid have also been explored for its cartilage regeneration potential. The incubation of chondrocytes with fragments of these gels in culture medium maintained their phenotype for 21 days, but the cells did not infiltrate into the gels as the pore size was in the range 200−2500 nm. The cells were tightly attached to the fragments, thus forming micromasses of combined cell/chitosan. A significant amount of neoformed extracellular matrix was accumulated between cells and hydrogel fragments, thus forming cartilage-like nodules. Pure chitosan physical hydrogel can thus be considered as a decoy of biological media, which is quite different from the scaffold.[85] Recent studies have shown that chitosan promotes attachment, proliferation, and viability of mesenchymal stem cells.[86] With these promising features, chitosan and its derivatives are considered as a very interesting biomaterial for use in cell transplantation and tissue regeneration.

**2.1.4. Alginate-Based Hydrogels.** Alginate is an unbranched binary copolymer of (1→4)-linked $\beta$-D-mannuronic acid and $\alpha$-L-guluronic acid (Figure 2). It readily forms bonds with a variety of divalent metal ions, such as calcium, magnesium, and barium, for example. This binding has been used to cross-link bulk alginates for a wide variety of applications, particularly in areas of tissue engineering. Ease of preparation, favorable cellular response, and low cost are advantages of alginate gels, making them attractive candidates for the development of tissue-engineering constructs. Alginate gel has been widely used for studying the phenotype, organization, and turnover of chondrocytes or intervertebral disk cells and the differentiation of adipose-derived adult stem cells and bone marrow-derived mesenchymal stem cells in a 3-dimensional system.[87−91]

Seeding chondrocytes in three-dimensional (3D) alginate cultures is reported to raise type II collagen expression significantly, and addition of BMP-2 has been shown to enhance this effect.[92] Combination of IGF-1 and BMP-1 has enhanced matrix synthesis by human adult normal and osteoarthritic chondrocytes cultured in alginate beads compared to those in which both growth factors were used alone.[93] Studies by Williams et al.[29]

indicated that initial cell density has a considerable effect on the composition, structure, and function of alginate−chondrocyte constructs and found that higher cell density and longer culture duration resulted in increased GAG and collagen content. Tensile moduli of the scaffolds seeded at 16 and 64 million cells/mL were, respectively, 45% and 116% greater than that of cell-free controls after 2 weeks.

One of the drawbacks of alginate gel is that cells suspended within the gel do not directly adhere to the matrix since there is no specific interaction between mammalian cells and the polysaccharide. Furthermore, alginate carries a negative charge balance and due to electrostatic repulsion proteins are not readily adsorbed. In order to improve adhesion of cells onto gels, alginate has been modified with peptides having an arginine−glycine−aspartic acid sequence (RGD). RGD functionalization has been found to enable chondrocytes to attach to alginate via integrins.[94] The extent of chondrocyte adhesion on RGD-functionalized scaffold was also dependent on the stiffness of the gel, which is controlled by the concentration of cross-linking agent. Gels cross-linked with 2.5−62.5 mmol $Ca^{2+}$/g alginate had moduli ranging from 12.3 to127 kPa, and maximum cell attachment (40%) was achieved on RGD substrates with modulus greater than 75 kPa.

Cai et al.[95] demonstrated that the bone marrow mesenchymal stem cells (BMMSCs) were well distributed in an alginate system after mixing and produced sufficient ECM in the implants to form chondroid aggregates. When implanted into nude mice after culture for 2 weeks, the experimental BMMSCs were observed to continue their process of induced chondrogenesis in vivo without supplemented factors in the inductive medium. High expression of aggrecan, SOX 9, and collagen type II demonstrated that the implanted cartilaginous constructs were mature and functional during chondrogenic BMMSCs terminal differentiation. Colemana et al.[96] found that rat BMMSCs expanded in monolayers in the presence of FGF-2 and media containing dexamethasone (10 nM) resulted in a higher accumulation of a cartilaginous matrix in alginate culture. Alginate gels containing both collagen type I and $\beta$-tricalcium phosphate were also found to enhance adherence and proliferation of human BMMSCs.[97] The response of bovine BMMSCs to peptides with arginine−glycine−glutamic acid sequence (RGE) and RGD-engineered alginate hydrogels was studied and found that while BMMSCs remained rounded and aggregated together on unmodified or RGE modified hydrogels, cells were rapidly adhered and spread on RGD-modified hydrogel surface. However, the interactions with RGD significantly inhibited the chondrogenic response to TGF $\beta$1 and dexamethasone, and inhibition increased with an increase in the bulk densities of RGD in the gel. It is suggested that the adhesion of cells to uniformly distributed RGD peptides in the alginate gel may prevent integrin clustering and signaling, thereby inhibiting the response to TGF-$\beta$1 and dexamethasone. Even though remarkable cell spreading was observed on 2D surfaces, the authors suggest that encapsulation of the BMMSCs in the 3D gel resulted in primarily rounded morphologies and potentially altered presentation of the RGD ligands, either of which may have subsequently influenced differentiation.[98]

Although alginate is an interesting scaffold material, the main disadvantage is its slow and uncontrollable degradation. This causes the release of high molecular weight constituents whose clearance from the body is limited. Bouhadir et al.[99] rectified this problem by preparing degradable gels by calcium cross-linking of periodate-oxidized alginate, which degraded within 9 days in PBS solution. The authors attributed the degradation of periodate-oxidized alginate to the formation of an open-chain adduct by cleavage of the carbon−carbon bond of the *cis*-diol group in the uronate residue, which could behave like an acetal group susceptible to hydrolysis. The partially oxidized alginate was capable of being ionically cross-linked with calcium ions to form gels. In vivo injection of these gels containing chondrocytes resulted in the appearance of native cartilage formation after 7 days. The constructs were highly cellular with uniform distribution, and there was no sign of an inflammatory response. On the other hand, in the case of unoxidized alginate-based hydrogels, small islands of cartilage-like tissue surrounded by significant amounts of residual alginate were found.

Variability of material properties depending on the source leading to a significant difference in chondrocyte proliferation, GAG production, and collagen type II expression is another potential drawback of alginate-based hydrogel systems.[100]

**2.1.5. Agarose-Based Hydrogels.** Agarose is a linear polysaccharide and consists of agarobiose repeat units, which are comprised of alternating units of galactose and 3,6-anhydrogalactose (Figure 2). Gelation of agarose occurs when a homogeneous solution is cooled from 99 to 35 °C, the temperature below which coil−helix transition takes place.[101] Agarose gel is one of the most frequently used systems for in vitro studies, particularly those involving mechanical stimulation, and has advantages of low cost, effective cellular responses, and easy preparation. Chondrocytes cultured in agarose gel preserve certain physiological features of chondrocyte behavior and have been used to investigate chondrocyte response to physical and chemical stimuli in a controlled manner.

There are numerous studies to demonstrate the suitability of agarose scaffolds for differentiation of stem cells into chondrocyte.[90,102,103] Bovine- and human-derived mesenchymal stem cells and adipose-derived stem cells were used in these studies.

Aulthouse et al.[104] found that human epiphyseal chondrocytes which lose their differentiated phenotype in the monolayer re-express the phenotype in an agarose gel within 1−2 weeks of culture. Studies by Ostensen et al.[105] showed that agarose gel could support the maintenance of normal and arthritic human chondrocytes in culture. Both normal and arthritic chondrocytes proliferated inside the gel and synthesized PG, but proliferation and PG synthesis occurred at a slower rate in chondrocytes from adult rheumatic patients than from healthy controls. Further, the ability of chondrocytes to synthesize and assemble a mechanically functional cartilage-like ECM in agarose gel was explored by Buschmann et al.[106] It was found that a matrix rich in PGs and collagen fibrils was developed around chondrocytes upon long-term culture of 40 days. While equilibrium modulus, dynamic stiffness, and oscillatory streaming potential rose to five times their initial values, the hydraulic permeability decreased to approximately 1/10th that of the cell-laden porous agarose at the beginning of the culture. The frequency dependence of the dynamic stiffness and potential obtained were suggestive of the formation of a mechanically functional matrix. The PG and protein synthesis were found to increase by 6−25% and 10−35%, respectively, by dynamic compression (3%, 0.01−1 Hz over 10 h),[107] whereas static compression inhibited cell proliferation and matrix synthesis of the cells grown in agarose.

Chondrocyte culture in the presence of synovial fluid in agarose gel has been shown to induce a state similar to that seen in vivo, with high levels of GAG synthesis.[108] It has been found

that a change in the extracellular pH could markedly affect the metabolic activities and biosynthetic ability of chondrocytes embedded in agarose gel, whereas total cell numbers and cell viability are not significantly affected.[40] Higher metabolic activities were observed at extracellular pH above the neutral point (pH 7.0), and even slight acidification (pH 6.6) clearly retarded energy metabolism. Mouw et al.[109] compared matrix composition and GAG fine structure among scaffolds based on agarose, alginate, collagen type I, fibrin, and poly(glycolic acid) and found that agarose constructs had the highest sulfated GAG to DNA ratio by 40 days of culture, suggesting that the matrix influences chondrocyte proteoglycan metabolism and may have an influence on the quality of tissue-engineered cartilage.

Studies of Ng et al.[110] showed that the application of dynamic physiologic loading to a bilayered chondrocyte-seeded agarose construct with a 2% (wt/vol) top layer and 3% (wt/vol) bottom layer over the 28 day culture period was found to significantly increase bulk mechanical and biochemical properties versus free-swelling culture. Even though preferential matrix formation was observed in 2% agarose layer, the matrix formed in the 3% agarose layer was more pericellularly localized. This is attributed to the effect of increased exposure of the top layer to mechanical stimuli during loading compared to the bottom layer. Hence, according to the authors the use of this type of bilayered construct to make depth-dependent inhomogeneity similar to the native tissue is not likely to be successful under long-term culture conditions. Mauck et al.[111] examined long-term maturation of bovine chondrocytes and mesenchymal stem cells (MSCs) in three-dimensional agarose culture. Their studies showed that even though chondrogenesis occurred in MSC-laden hydrogels, the amount of matrix formation and mechanical properties were lower than that produced by chondrocytes under the same conditions. In addition, GAG content and equilibrium modulus plateau with time in MSC-laden constructs, indicating that the diminished capacity was not a result of delayed differentiation. Further optimization is required to achieve levels similar to those produced by chondrocytes by using MSCs in agarose gel. Recent studies demonstrated that the tensile properties of cell-seeded agarose constructs increased with the increase in the duration of culture and application of TGF-$\beta$3. The increase in tensile properties obtained was similar for both calf chondrocytes and calf bone marrow-derived MSCs-seeded constructs when cultured at the same seeding density. However, the constructs did not have tensile properties matching with native cartilage.[112] Poor mechanical strength is the main disadvantage of agarose-based gels. There is a need for further improvement of these engineered constructs to facilitate their load-bearing capacity once implanted in vivo.

## 2.2. Protein-Based Hydrogels

Many of nature's scaffolds and templates are protein based. Scaffolds formed from proteins are particularly suited for tissue engineering as they comprise the structural basis for extracellular matrices of tissues. Moreover, in protein scaffolds, cell adhesion molecules (integrins) can directly bind to the scaffold material. Fibrous proteins (collagens, silks) in particular are attractive, due to their architectural features and impressive mechanical properties. These materials are often studied as porous scaffolds as well as in fiber forms for ligaments and tendons. Like polysaccharides, proteins also can be cross-linked to form hydrogels.

Elastin, an ECM protein, has received less attention because of very complex purification methods. However, recombinant systems have been developed to produce elastin-like peptides that can self-assemble in response to pH, temperature, and ionic strength. These molecular-engineered peptides can self-assemble into stable hydrogels without using any cross-linking agents and have been explored for cartilage tissue engineering.[113] Among the different protein-based hydrogels which are of natural origin, those based on collagen/gelatin, fibrin, and silk fibroin have been exploited most frequently for cartilage tissue engineering and are described in detail in this section.

**2.2.1. Fibrin-Based Hydrogels.** Fibrin, the structural component of blood clots, has been utilized for different applications in the field of tissue engineering, as it fulfills specific physical and biological requirements. Formation of fibrin involves different steps, initially the cleavage of small peptides such as fibrinopeptides A and B present in fibrinogen molecule by the serine protease, thrombin. The cleaved fibrinogen, which is called fibrin monomer, has certain binding sites which then interact with the complementary binding sites of other fibrin monomers forming oligomers with half-staggered overlapping and further aggregate laterally to form fibers and a three-dimensional network.[114]

The main advantage of fibrin hydrogels is the ability to form injectable scaffolds which are minimally invasive for cartilage tissue engineering.[115] Series of studies conducted in swine knee model defects demonstrated that fibrin gel is a suitable polymer gel for generating new cartilage matrix, capable of forming mechanical bonds between cartilage disks resulting in healing and integration. The main disadvantage of fibrin is its increasing instability and solubility over time in vitro and in vivo due to fibrinolysis. However, it has been reported that by varying the fibrin parameters, such as fibrinogen concentration, thrombin concentration, and ionic strength, one can generate gels with different appearance, mechanical properties, and stability.[116−118] Composite grafts of hydroxyapatite loaded with chondrocyte fibrin suspension have been studied for the treatment of large cartilage defects in a goat model. Hydroxyapatite was incorporated to provide stability as fibrin gel alone was not able to re-establish both the entire subchondral cancellous bone defect and the covering articular joint surface.[119] The longevity of fibrin can be extended by the use of fibrinolysis inhibitors, primarily protease inhibitors such as aprotinin or tranexamic acid that are added to the fibrin gel and/or as a supplement to the cell culture medium, which can retard degradation, thereby partially stabilizing the fibrin gel for 4 weeks.[120−122] However, resultant stability was still inadequate for applications where long-term shape stability is desired.

Eyrich et al.[123] tried to optimize fibrin gel which is stable in culture medium even without fibrinolysis inhibitors. They found that gels are stable and transparent with an increase in the $Ca^{2+}$ concentrations from 2.5 to 20 mM and at pH 6.8 and 9. Their studies revealed that chondrocyte seeding (75 million cells/mL) within these gels resulted in the formation of an engineered tissue with increased wet weight, high fractions of GAG (approximately 4.5%) and total collagen (approximately 6%) per weight after 5 weeks. Further, fibrin sealent (Tisseel), a biological adhesive material, recently recommended as an adjunct in autologous chondrocyte implantation (ACI), has been found to support both the migration and the proliferation of human chondrocytes. Kirilak et al.[124] proposed that these effects are mediated, at least in part, via thrombin-induced PAR-1 signaling in human chondrocytes.

**2.2.2. Collagen/Gelatin-Based Hydrogels.** Collagen is a biological macromolecule abundant in cartilage tissue, and as a result it has been tested as a carrier material in tissue-engineering

applications. The main disadvantage of collagen is its significant degradation rate, which leads to loss of mechanical properties before healing is complete.[125] Cells in collagen can bind to the scaffold via integrins and thus play an important role in signal transduction pathways. Collagen type II has several advantages and has been reported to act as a physiological articular cartilage matrix, initiate and maintain MSC chondrogenesis, and enhance the effect of TGF $\beta$1.[126] While collagen type II is the main component of extracellular matrix of cartilage, collagen type I is often used for tissue engineering alone or in combination with other polymers.[127,128]

Collagen type I is a fibrillar protein usually found in diseased or damaged articular cartilage and has the advantage of spontaneously polymerizing into a stable gel at neutral pH and physiologic temperatures. Despite the foreign nature of the type I collagen environment, studies revealed that articular chondrocytes remain viable and can secrete cartilage-specific matrix components[129−138] responding to mechanical stimuli when cultured in those gels. Galois et al.[139] demonstrated that the cellular and biochemical composition of neocartilage formed are highly dependent on culture conditions, such as the use of floating or attached gels, collagen gel concentrations, or initial cell density. Chondrocyte phenotype after 2 weeks of culture in collagen gels under floating condition was found to be better maintained rather than attached conditions. This suggests the usage of attached gels to permit chondrocyte proliferation initially and then release of the gel causing its contraction leading to the formation of a stable and dense matrix with a final size tailored to the cartilage defect present in the patient. Gels containing chondrocytes and collagen type I used to repair full-thickness defects in the articular surface of small animals were found to impart moderate regeneration of the articular surface.[140] There were attempts to label human mesenchymal stem cells using very small super paramagnetic iron oxide particles embedded in collagen type I hydrogel for visualizing the cells in the matrix after implantation using magnetic resonance imaging. Labeling was not found to affect the chondrogenic differentiation of cells.[141] Nehrer et al.[134] compared the behavior of chondrocytes in type I and type II collagen matrices and found a dramatic difference in the percentage of cells displaying spherical (chondrocytic) or elongated (fibroblastic) cell morphology in the two matrices. When 60% of cells in collagen type II sponge retained its spherical shape after 14 days of culture, only 30% cells were in spherical shape in type I collagen.[134] Studies of Buma et al.[142] showed that a composite matrix consisting of a deep layer of type I collagen for subchondral recruitment of progenitor cells and a more superficial layer of type II collagen for maintenance of the chondrocyte phenotype may be the matrix of choice for collagen-based regeneration of full-thickness articular cartilage defects.

Gelatin, the denatured collagen, has also been explored widely for its cartilage regenerative efficacy. Genipin cross-linked gelatin showed collagen and GAG production in 9 days, and a tissue with a cell distribution resembling that of the native cartilage was developed after 30 days cell culture.[143] The main drawbacks of collagen-based gels include limited availability, high cost, low mechanical strength, and shrinkage of gels during culture.[139]

**2.2.3. Silk-Based Hydrogels.** Silks, naturally occurring polymers extruded from insects or worms, are composed of a filament core protein, termed fibroin, and a glue-like coating consisting of sericin proteins. Recently, silk fibroin has been increasingly studied for many biomedical applications due to its biocompatibility, slow degradability, and remarkable mechanical properties.[144] Biodegradation of silk by ubiquitous proteases has been reported over days to years depending greatly on the animal model, implantation site, and formulation of silk protein. Intramuscular (short,term; 8 weeks) and subcutaneous implantation (long term; 1 year) on lewis and nude rats revealed that aqueous-derived silk fibroins were found to degrade faster compared to hexafluoroisopropanol-(HFIP)-derived ones. Aqueous-derived ones degraded completely between 2 and 6 months, while those derived from HFIP persisted beyond 1 year.[145,146] Surface modification techniques and the ability to control molecular structure and morphology have further expanded its potential. Also, silk fibroin has been shown to support stem cell adhesion, proliferation, and differentiation in vitro and promote tissue repair in vivo. Fibroin was derived previously from *Bombyx mori* silkworm. However, silk fibroins from spiders and those formed via genetic engineering or the modification of native silk fibroin sequence chemistries provide new options to further expand the utility of silk fibroin-based scaffolds for tissue-engineering applications. Hydrogels based on silk fibroins are also prepared by sol−gel transition in the presence of acid, ions, or other additives.[147,148] The other factors that affect the gelation reaction are temperature, concentration of fibroin, pH, and concentration of additives such as $Ca^{2+}$, glycerol, and poly(ethylene oxide).[149,150]

Silk fibroin hydrogel-derived sponges have been combined with freshly isolated rabbit chondrocytes for in vitro cartilage tissue engineering.[151−153] It was found that chondrocytes proliferated and maintained the differentiated phenotype in silk fibroin. Dynamic viscoelasticity measurements performed under compressive loading revealed that dynamic modulus and the dynamic loss of the regenerated cartilage increased and the peak value of tan delta as well as the frequency at the peak decreased with increasing cultivation time. The authors attribute the changes in the dynamic viscoelastic properties of the regenerated cartilage to narrowing of the fluid path by synthesized extracellular matrix.[154] Meinel et al.[155] compared the features of newly constructed cartilage derived by engineering human MSCs on unmodified and cross-linked collagen scaffolds and silk fibroin scaffolds. Stable, slow degrading scaffolds like cross-linked collagen scaffolds, silk, and RGD-modified silk scaffolds were found to maintain sufficient cell density and promote the formation of cartilage-like extracellular matrix, as evaluated by total DNA content and GAG deposition. It was found that MSCs in the porous silk fibroin scaffolds deposited higher amounts of cartilage-specific ECM proteins (GAGs and collagen type II) and expressed higher levels of collagen type II mRNA than MSCs in the collagen-based scaffolds after 4 weeks. Evaluation of attachment, proliferation, and differentiation of MSCs in the silk scaffold revealed that after 3 weeks of cultivation, the spatial cell arrangement and the collagen type-II distribution in the MSCs−silk scaffold constructs resemble those in native articular cartilage tissue, suggesting promise for these novel 3-D degradable silk-based scaffolds in MSC-based cartilage repair.[156] Further in vivo evaluation needs to be performed to evaluate the clinical relevance of silk-based scaffolds for cartilage regeneration.

## 3. HYBRID HYDROGELS

Though hydrogels derived from biopolymers exhibit remarkable advantages over synthetics, especially with respect to cell signaling, they lack versatility in designing exogenous ECM with specific properties (such as porosity and mechanical strength).

Synthetic polymers, on the other hand, can be manufactured reproducibly on a large scale and processed into exogenous ECM in which one can readily control and manipulate the macrostructure, porosity, mechanical strength, and degradation time. However, a disadvantage of the synthetic polymers is the lack of cell recognition signals. The concept of combining synthetic materials with cell-recognition sites of naturally derived biomaterials has received great attention. These hybrid materials could possess the favorable properties of synthetic materials, such as controllable mechanical and degradation properties, reproducible large-scale production, and good processability, as well as the specific biological activity of naturally derived materials. Hybrid hydrogels using a combination of different biopolymers also have been explored for imparting mechanical strength.

### 3.1. Hybrid Scaffolds Using Synthetic Polymers along with Biopolymers

Three-dimensional poly(alpha ester) sponges are relatively hydrophobic, but hybridization with collagen microsponges increased their wettability and facilitated cell seeding, resulting in a spatially uniform cell distribution throughout the hybrid matrix. It was found that bovine articular chondrocytes proliferated, regenerated the cartilaginous matrix, and maintained their phenotypical round morphology after culturing for 6 weeks in the poly(lactic-*co*-glycolic acid)−collagen hybrid matrix. They also showed a higher mechanical strength than either poly(alpha ester) or collagen scaffolds.[157,158] Fan et al.[159] evaluated the potential of novel hybrid poly-(lactic-*co*-glycolic acid)−gelatin/chondroitin/hyaluronate (PLGA-GCH) scaffolds in cartilage repair. It was found that differentiated MSCs seeded on PLGA-GCH significantly increased the proliferation of MSCs and GAG synthesis compared to PLGA scaffolds. Similarly, Lee et al.[160] investigated the use of a fibrin hydrogel to improve the potential of a polyurethane scaffold-based system for articular cartilage tissue engineering. It has been reported that use of fibrin could allow high viable cell seeding efficiency and homogeneous cell distribution, an increase in the percentage of newly synthesized GAG and type II collagen, and aggrecan gene expression through day 14.

Reports showed that chitosan can enhance chondrocyte attachment to poly(L-lactic acid), cell adhesion, proliferation, and biosynthetic activity.[161,162] Hydrogels prepared by derivatizing chondroitin sulfate and then copolymerizing with methacrylated PEG forms a unique combination of synthetic as well as natural component. With the incorporation of 40% CS−methacrylate into the copolymer network, the compressive modulus increased 4-fold compared to pure PEG gels with statistically similar swelling ratios values (9.3). In addition, gels with a swelling ratio of 7.5, sufficient for proteoglycan diffusion in pure PEG gels, were obtained by incorporating 25% CS−methacrylate into the network. Gene expression studies confirmed these results, showing significantly higher collagen type II expression in PEG/CS copolymer gels compared to pure CS gels after 2 weeks.[69]

Hybrid scaffolds prepared by the physical cross-linking of poly(vinyl alcohol) (PVA) followed by chemical cross-linking with alginate exhibited highly porous, open-cellular pore structures with almost the same surface and cross-sectional porosities (85%) and a pore size from about 290 to about 190 $\mu$m with increasing PVA composition. These scaffolds were softer and more elastic than the control alginate scaffold without affecting the mechanical strength. These hybrid scaffolds showed better cell adhesion and faster growth than the control alginate scaffold.[163] Jin et al.[164]prepared PLGA/alginate hybrid to exploit mechanical support and stability provided by PLGA, improved loading and retention of cells offered by alginate gels, and found the presence of cartilage-specific matrix after 4 and 12 weeks in vivo culture.

### 3.2. Hybrid Scaffolds Using Combination of Biopolymers

Combinations of different biopolymers also have been exploited for the development of suitable scaffolds for cartilage tissue engineering. Hydrogel particles of HA have been covalently coupled to photo-cross-linkable HA hydrogels (HA-MA) to develop hierarchically structured, mechanically robust, and biologically active hydrogels suitable for cartilage tissue engineering. Compared to conventional HA-MA hydrogels, these hydrogels contained lower sol fraction, swelled to a lesser extent, and were more enzymatically stable.[165] In order to provide mechanical strength, gelatin/hyaluronicacid/chondroitin-6-sulfate (GHC6S) tricopolymer matrix has been added into fibrin glue.[166] Histological examination revealed that the chondrocytes cultured in GHC6S-fibrin glue showed a round shape with intact lacuna structure and a homogeneous distribution indicating that hyaluronic acid within the particles helped the migration and proliferation of chondrocyes. Chondrocytes cultured in GHC6S-fibrin glue effectively promoted ECM secretion and inhibited ECM degradation. Chang et al.[167] prepared a tricopolymer scaffold of CS, HA, and gelatin by cross-linking using water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)) which can activate carboxylate groups to form amides or esters, to mimic the cartilage matrix. Hydrogels prepared were porous in nature with a pore size of 180 $\mu$m and a porosity of 75% suitable for penetration, growth, and proliferation of cells. Secretion of extracellular matrix was seen only after 4 weeks of culture, and lacuna formation seen in the matrix surrounding the chondrocytes was similar to that of natural cartilage. Immunohistochemical staining for S-100 protein and type II collagen showed that chondrocytes cultured in spinner flasks for 5 weeks retained their phenotype in this tricopolymer scaffold and stained positive for S-100 protein. Chondrocytes maintained their functionality inside the matrix by producing type II collagen. However, the amount of synthesized matrix was not enough to form cartilage, which could be due to inhibition of cell mitosis by CS, and higher ECM secretion with lacuna formation and more GAG production are expected after longer periods of culture.

Studies have shown that the combination of growth factors and a porous scaffold might substantially improve the cartilage forming efficacy. Lee et al.[168] developed porous collagen/chitosan/CS scaffold by cross-linking with EDC, and growth factor-loaded chitosan microspheres were incorporated into this scaffold to achieve a controlled release of growth factors which can enhance chondrogenesis. The scaffold had interconnected pores of mean diameter 195.7 ± 44.2 $\mu$m, and GAG synthesis by chondrocytes encapsulated in the matrix increased with time. The TGF-$\beta$1 microspheres caused a significant increase in GAG production after 2−3 weeks of in vitro incubation. The dry weights of constructs from the TGF-$\beta$1 group increased significantly after culture, which implies that tissue-engineered cartilage formation may be effectively stimulated by TGF-$\beta$1-loaded collagen/chitosan/GAG scaffolds. However, in this study, the effects of the presence of TGF-$\beta$1-loaded chitosan microspheres are only detected after 14 and 21 days in culture, and these late effects could be explained by the modest mitotic property of chitosan. Chitosan/alginate/hyaluronan complexes

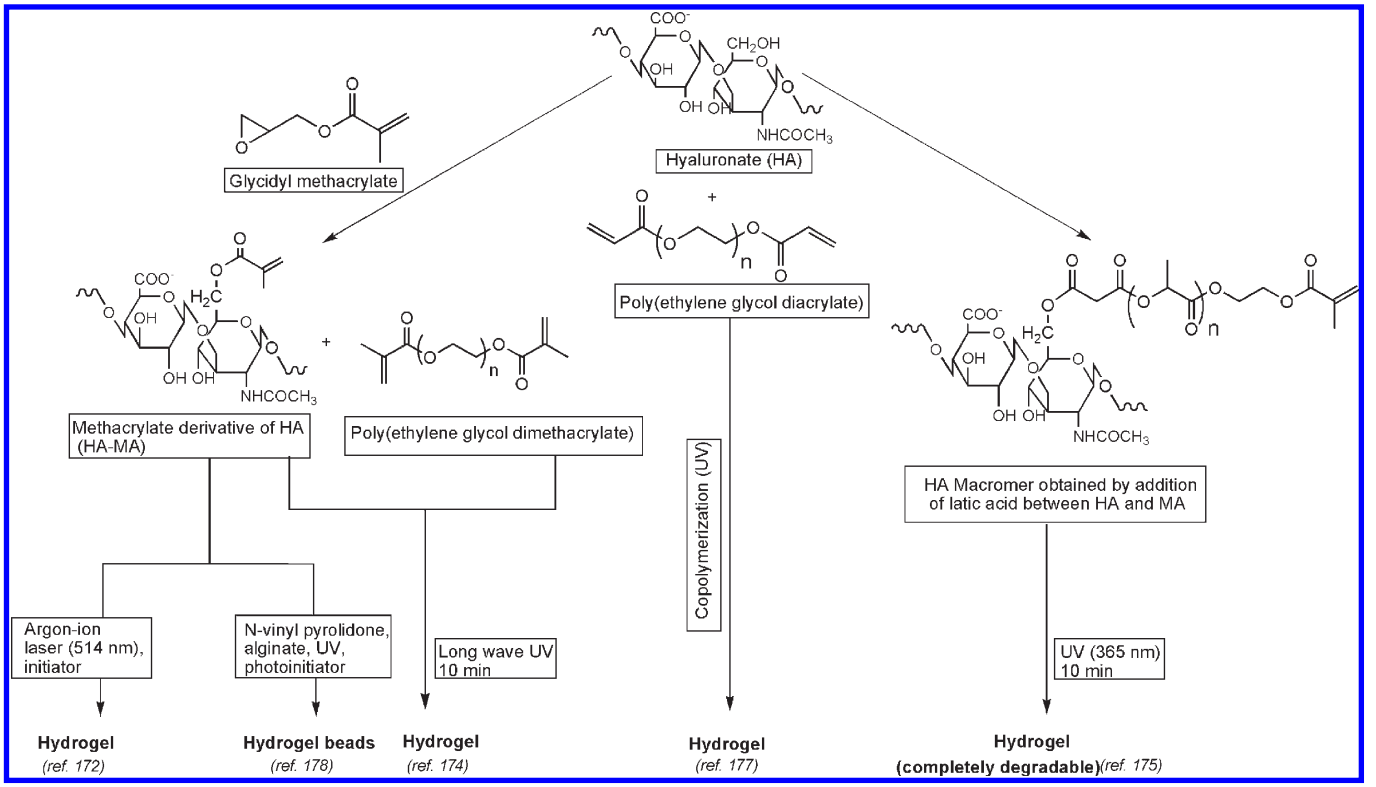


**Figure 3.** Strategy for the preparation of injectable hydrogel via photo-cross-linking from hyaluronate.

with or without covalent attachment with RGD-containing protein have also been reported to provide partial repair when implanted into rabbit knee cartilage defects.[169]

## 4. INJECTABLE HYDROGELS

Injectable scaffolds are superior to preformed scaffolds in terms of improved patient's compliance, ease of clinical implementation for the treatment of geometrically complex, and large lesions via minimally invasive techniques such as arthroscopy.[170,171] Natural polymers such as collagen, chitosan, gelatin, alginate, and hyaluronan have been utilized for the development of injectable systems. Fibrin and alginate-based hydrogels are the most extensively studied injectable systems. Different strategies to make in situ gelling systems from polymers include photo-cross-linking, chemical cross-linking, enzymatic cross-linking, pH-induced gelation, temperature-induced gelation, ionic interaction, and hydrophobic interactions. The rate of cross-linking and conditions under which cross-linking reaction occurs determine the usefulness of each cross-linking method. Cross-linking should occur in such a way that the liquid solution of the polymer with cells can be injected to fill any irregular defect and can be polymerized in situ within a short period of time to form a solid gel. The temperature and pH of the polymer solution should be appropriate so that cells will have maximum viability. Cross-linking should not be too fast; otherwise, the syringe needle used for injection may become clogged prior to instillation.

### 4.1. Injectable Hydrogels via Photo-Cross-Linking

The basic principle of this method includes the introduction of polymerizable vinyl groups onto the polymer using certain chemicals (for example, methacrylol chloride, glycidyl methacrylate, methacrylic anhydride), and polymerization occurs when exposed to UV−vis light in the presence of some acceptable initiators (for example, camphorquinone, Irgacure 2959 (I2959)) (Figures 3 and 4). There are also studies on copolymerization of polymer with acrylic monomers resulting in in situ gelling systems. The concentration and type of the photoinitiators should be controlled in such a way that the photoirradiation will not produce any unwanted reactions like tissue necrosis due to heat generation and cell toxicity.

Various strategies adopted for the development of injectable gels from HA are summarized in Figure 3. Injectable gels have been prepared from a photo-cross-linkable HA derivative of methacrylate (HA-MA) and evaluated for articular cartilage repair.[172] The solution of the derivative (2%) had a low viscosity, easily mixed with cells in vitro, and readily filled irregularly shaped defects in vivo. Cross-linking of the solution occurred rapidly on exposure to light, resulting in the formation of a three-dimensional hydrogel of considerable mechanical integrity. It was found that photo-cross-linkable HA-MA promoted retention of the chondrocytic phenotype and cartilage matrix synthesis for encapsulated chondrocytes in vitro and accelerated healing in an in vivo osteochondral defect model. At higher concentrations of derivative (5%), enhanced chondrogenesis, increased expression of cartilage-specific matrix, and increased accumulation of proteoglycan were observed, which the authors attribute to the greater probability of receptor-mediated interactions.[173] Similarly, Burdick et al.[174] encapsulated primary auricular swine chondrocytes in photo-cross-linkable (initiator-I2959; long-wavelength UV; intensity ≈ 4 mW/cm$^2$; irradiation time = 10 min) HA-MA, implanted subcutaneously on nude mice, and neocartilage formation was studied for a period of 8 weeks. The results were compared with poly(ethylene glycol dimethacrylate) (PEGDM) hydrogels, which are extensively studied as


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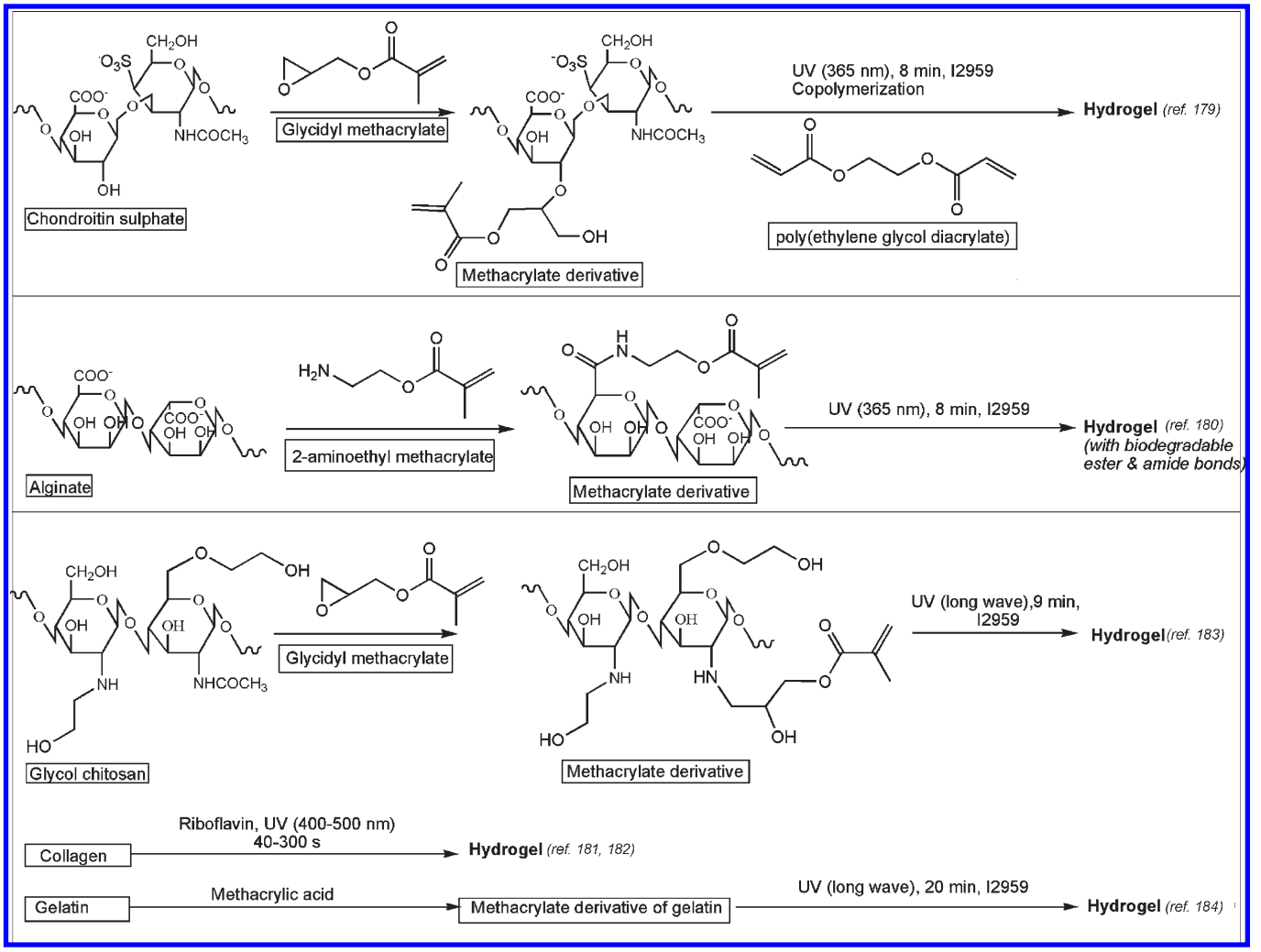


**Figure 4.** Strategy for the preparation of injectable hydrogel via photo-cross-linking from chondroitin sulfate, alginate, chitosan, collagen, and gelatin.

photo-cross-linked chondrocyte carriers. GAG formation was more uniformly distributed in HA-MA hydrogels than in non-degradable PEGDM hydrogels, which actually prevent the distribution of large ECM molecules, demonstrating that the cell-controlled degradation of HA-MA hydrogels is crucial in optimizing neocartilage formation and distribution. HA-MA hydrogels are degraded in the body by the enzyme hyaluronidase, which if not present in adequate quantities can hinder diffusion of growth factors, migration of cells, and distribution of extracellular matrix proteins, and the degradation product will be modified HA rather than potentially biologically active unmodified HA. It has been found that by incorporating hydrolytically degradable repeating units of α-hydroxy esters (e.g., lactic acid) between the HA and the polymerizing methacrylate moiety hydrolytically degradable HA hydrogels (initiator, I2959; UV 365 nm; intensity $\approx$ 2 mW/cm$^2$; irradiation time = 10 min) can be prepared. Complete degradation into HA, lactic acid, and chains of poly-(methacrylic acid) was found for such hydrogels even without hyaluronidase within 9 days.[175]

Injectable, in situ cross-linkable HA hydrogels synthesized by cross-linking of thiolated HA with PEG diacrylate (gelling time 10 min) were found to be as biointegrative materials for tissue engineering. However, no studies have been reported to prove its potential as a cartilage tissue-engineering scaffold.[176] Studies conducted on mesenchymal cells encapsulated in hydrogels prepared by photo-cross-linking of HA with PEG−diacrylate coencapsulated with TGF-$\beta$-3 showed cartilage differentiation.[177] Another approach studied for cartilage tissue engineering is injectable chondrocytes encapsulated hydrogel beads based on HA exploiting the advantage of a large surface-to-volume ratio for increasing the mass transfer rates.[178] Hydrogel beads prepared by photopolymerization of methacrylated HA and *N*-vinyl pyrrolidone (long-wavelength UV, intensity = 7 W/cm$^2$) using alginate as a temporal spherical mold were degradable in the presence of hyaluronidase enzyme. Viable cells were injected into the preformed beads by the microinjection technique. The limitation of such a system is the creation of a defect by the needle used for injection and low cell seeding efficiency as a considerable portion of the cells flows out of the bead. Though poly(lysine) was used to seal the physical defect created, only 35 443 $\pm$ 5360 cells could be seeded in such a treated bead. Neverthless, poly(lysine)-treated HA beads exhibited a significant increase in proliferation rate compared to that of the untreated HA beads.

Photo-cross-linkable hydrogels with good mechanical strength from CS were prepared by polymerization of a methacrylate derivative of CS and its copolymerization with poly(ethylene


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oxide diacrylate) (PEODA) in the presence of UV photoinitiator I2959 using an UV light source (intensity ≈ 10 mW/cm$^2$) at a wavelength of 365 nm.[179] These gels degrade in the presence of chondroitinase enzyme in a dose−response manner, whereas no degradation was observed in the absence of the enzyme. Gels were compatible with chondrocytes, but more detailed studies are required to prove its suitability for cartilage tissue engineering. Similarly, methacrylate derivatives of alginate have been synthesized by its reaction with 2-aminoethyl methacrylate (AEMA) and photo-cross-linked in the presence of I2959 initiator with UV light at 365 nm at intensities of 8−20 mW/cm$^2$ for 8 min to form hydrogels.[180] The advantage of using AEMA is that it imparts biodegradable linkages within the hydrogels which are susceptible to hydrolysis. Viability studies on chondrocytes demonstrated the nontoxic nature of hydrogels and degradation products. Detailed evaluation is needed for substantiating the potential of such hydrogels on cartilage tissue engineering.

Collagen hydrogels were prepared by photo-cross-linking in the presence of riboflavin (type II photoinitiator) on exposure to visible light (440−500 nm, 0.5 W/cm$^2$) for 40−300 s.[181,182] Implantation studies of chondrocytes encapsulated within these hydrogels demonstrated hyaline-type cartilage formation and integration of newly formed cartilage with the native tissue within 6 weeks. The authors attribute the host tissue integration to the molecular interactions with the native cartilage surrounding the lesions during in situ cross-linking of collagen in the presence of riboflavin. Methacrylation of glycol chitosan yields hydrogel upon photo-cross-linking in the presence of I2959 on exposure to an UV source (320−480 nm) at intensities ranging from 25 to 102 mW/cm$^2$ for 9 min. These gels were degradable in the presence of lysozyme and compatible with chondrocytes. The Young's moduli obtained for these hydrogels (0.44 MPa) were lower than that for human tibial articular cartilage (1−10 MPa), showing the need for further modification of the system to improve the mechanical strength.[183] Photo-cross-linkable gelatin hydrogels were prepared by derivatization with methacrylic acid followed by UV irradiation (365 nm) in the presence of I2959 for 20 min.[184] It was found that the hydrogel supported chondrocyte growth and maintained chondrocyte phenotype under in vitro culture conditions for 12 days.

The advantage of photo-cross-linking is the ease with which different chemistries can be integrated into the hydrogel by mixing selected derivatized biopolymers followed by copolymerization. Moreover, it has better spatial and temporal control over gelation. However, the drawback of photo-cross-linking is the requirement of a photoinitiator, which may have toxic effects and prolonged irradiation time, leading to a local temperature increase that can subsequently harm neighboring cells and tissues, limiting their wide use.[185]

### 4.2. Injectable Hydrogels via Chemical Cross-Linking

Chemical cross-linking of polymers is also exploited for the development of injectable cartilage tissue-engineering scaffolds. The advantage of using chemical cross-linking is a precise control over mechanical stability and degradability of hydrogels. Further, the reaction can be manipulated by careful selection of the concentration of the polymer solution and cross-linker to obtain the desired gelling time as required. Moreover, using suitable carriers (for example, liposomes), triggered release of cross-linker can be achieved, preventing premature gelation in otherwise rapidly gelling fibrin and alginate-based hydrogels.[186] Various strategies used for the development of such injectable scaffolds are summarized in Figure 5. Among them, fibrin and alginate-based hydrogels are more widely studied as injectables. The chemistry behind hydrogel formation of these polymers has already been discussed in the previous section. The potential of fibrin hydrogel alone for cartilage regeneration is limited by its own degradation and lack of mechanical stability. Wei et al.[187] reported that adipose-derived adult stem cells cultured in fibrin−chondroitin−sulfate matrices are more useful in promoting and retaining many characteristics of chondrogenic phenotype compared to the fibrin alone. Alginate, as a liquid, is injected and cross-linked with calcium to prevent migration and allowed the formation of a tissue with similar morphological characteristics as the native hyaline cartilage. However, the reversible and toxic nature of the metal ions limits its wide applicability.[188] Self-cross-linking biopolymers without using any cross-linking agents can address such issues.[189]

Long-term implants using alginate scaffolds are limited as the scaffolds lose their functionality within a year due to their reversible nature, leading to insufficient mechanical integrity. Shear reversibly cross-linked hydrogels that can recover gel structures from shear-induced breakdown have been prepared by Park et al.[190] from alginate in order to deliver primary chondrocytes and to regenerate cartilage tissue formation in vivo. These gels, which can be injected as liquid but regel once placed in the body, were prepared by the combination of calcium cross-linking and cell cross-linking techniques. Cell interactive polymer was prepared by the covalent coupling of RGD peptides to alginate, which in the presence of cells can form gels without the use of any external cross-linking agents. Such a hydrogel shows shear reversible gelation behavior because of reversible weak binding of RGD peptides coupled to alginate backbone to the integrin receptors. Disadvantages of such a technique include the weak physical nature, large number of cells ($1.25 \times 10^8$ cells/mL) required for cross-linking, and resulting necrosis due to an insufficient supply of nutrients and oxygen upon delivery into the body. The number of cells used for cross-linking has been reduced to some extent by using a low concentration of calcium ions.

Injectable hydrogels were also prepared via a Michael-type addition reaction of thiolated HA and 4-arm poly(ethylene glycol)-vinyl sulfone.[191] Gelation time was found to decrease with the increase in the concentration of polymer precursors and molecular weight (45−185 KDa) of HA used, and the shortest gelation time obtained was 1 min. The degradation time of hydrogels was found to increase (15 days) with the molecular weight of HA and its degree of substitution. Degradation studies in the presence of chondrocytes revealed no degradation until the seventh day and afterward a fast degradation observed by days 14 and 21 which the authors attribute to the production of hyaluronidase enzyme by the incorporated chondrocytes. Chondrocyte proliferation estimated by DNA analysis was found to increase during the first week of culture and thereafter due to gel degradation, cells released into the medium decreasing the DNA content per gel. There are reports on injectable chemically cross-linked hydrogels prepared by radical polymerization of methacrylic and lactic acid-grafted chitosan. Polymerization was initiated by the redox system comprising of an oxidant (ammonium persulfate) and reducer (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine), and hydrogel was formed after 8 min.[79] Chondrocytes were found to survive in these hydrogels with normal morphology through culture duration of 12 days.

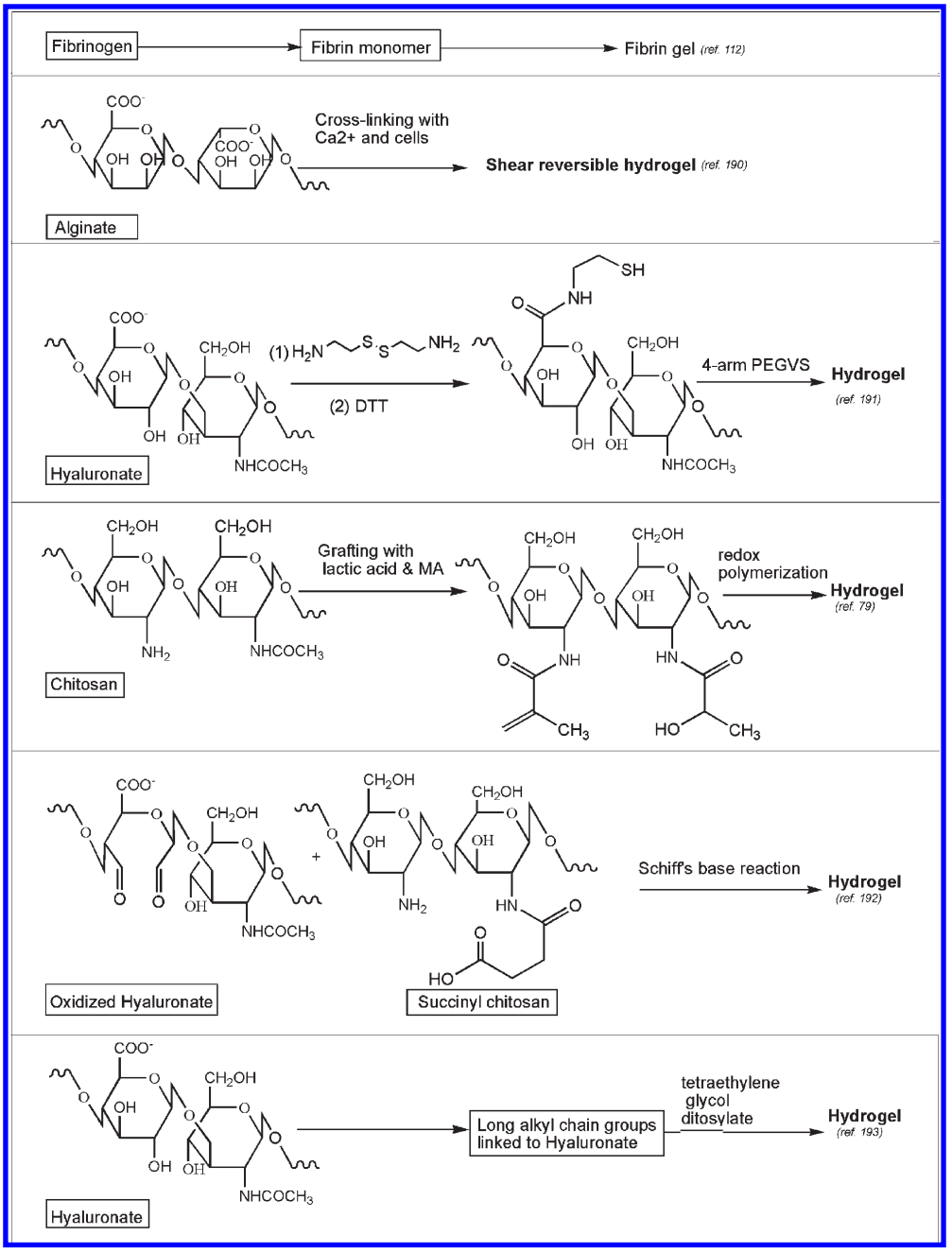


**Figure 5.** Strategy for the preparation of injectable hydrogel via chemical cross-linking.

Injectable in situ forming biodegradable composite hydrogels based on chitosan and HA were prepared by using the Schiff's base reaction between aldehyde groups of oxidized HA and amino groups of succinyl chitosan.[192] Succinyl derivative was synthesized to make the chitosan soluble at physiological pH. The concentrations of both succinyl chitosan and oxidized HA were varied to prepare different hydrogels (gelling time 1−4 min), and it was found that degradation is slow for those hydrogels prepared with more succinyl chitosan than oxidized HA. About 93% of encapsulated chondrocytes survived after 24 h of culture. Mechanical properties are to be improved further.

Huin-Amargier et al.[193] introduced long-chain alkyl groups onto HA, exploiting the beneficial aspects of hydrophobic interaction of long alkyl chains which can lead to the formation of 3D networks for cartilage repair. HA was substituted by aliphatic chains with 12 carbon atoms, which as such can give rise to 3D networks, and on the other hand, the same was then cross-linked with a chemical reagent tetraethylene glycol ditosylate. Reaction parameters were optimized in such a way that both physically and chemically cross-linked hydrogels demonstrated improved rheological properties as compared to those of only physically cross-linked gels obtained from amphiphilic derivatives. Similarly, hydrogels were also prepared by introducing aliphatic chains with 18 carbon atoms and explored for its suitability as an injectable cartilage engineering scaffold.[194]


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### 4.3. Injectable Hydrogels via Enzymatic Cross-Linking

Gelation using enzymatic cross-linking has the advantage of providing good control over reaction rates under mild reaction conditions and high biocompatibility.[195] Peroxidases (particulary horseradish peroxidase (HRP)) catalyze cross-linking through oxidative polymerization of phenol derivatives in the presence of $H_2O_2$ (Figure 6).[196] Biodegradable injectable chitosan hydrogels were obtained from water-soluble chitosan derivatives (chitosan-graft-glycolic acid and phloretic acid) through enzymatic cross-linking with HRP and $H_2O_2$.[197] Gelation occurred within 4 min. Gels were compatible with chondrocytes but not completely degradable within 1 month in the presence of lysozyme. Injectable biomimetic hybrid hydrogels prepared by HA grafted with dextran−tyramine conjugate also showed enhanced chondrocyte proliferation and matrix production.[198] It is the tyramine residues that enabled the enzymatic cross-linking of the HA and dextran in the presence of HRP and $H_2O_2$ leading to gel formation within 1 min. In the presence of hyaluronidase, gels were stable with more than 30 wt % remaining after 21 days of degradation. Injectable gels prepared using HA-tyramine conjugates by Kurisawa et al. were found to be biocompatible, biodegradable, and suitable for drug delivery and tissue-engineering applications. However, this system has not been evaluated for its cartilage regenerative efficacy.[195] While enzymatic cross-linking has been used for the development of hydrogels, very few systems have been exploited for their use as injectable scaffolds in cartilage tissue engineering. This can be attributed to potential cytotoxic effects exerted by a local high concentration of $H_2O_2$ before homogeneous mixing during injection.[199]

### 4.4. Injectable Hydrogels via Temperature Change

Certain polymers do not require organic solvents, copolymerization agents, or an externally applied trigger for gelation but rather will undergo phase transition with a change in temperature, leading to the formation of hydrogels. The sensitiveness of molecular interactions like hydrogen-bonding and hydrophobic interactions to temperature contribute much to such phase separation. At the lowest critical solution temperature, hydrogen bonding between polymer and water weakens and polymer−polymer interaction predominates leading to the abrupt dehydration of the macromolecule and becomes more hydrophobic.[200] Amphiphilic polymers self-assemble in solution, leading to micelle packing and gel formation as polymer−polymer interaction increases with the increase in temperature.[201] Selection of biopolymer derivatives are to be carefully done so that the precursor solution should be liquid at ambient temperature and should gel at 37 °C.

Hydrogels prepared by temperature-induced gelation are summarized in Figure 7. Hydrogel blends of HA with thermosensitive poly(*N*-isopropyl acrylamide-*co*-acrylic acid) have been studied as an injectable tissue-engineering construct for cartilage repair. Blend hydrogel was incorporated with dexamethasone and growth factor TGF $\beta$-3 in order to enhance chondrogenic differentiation and injected to the back subcutis of mice. Low molecular weight dexamethasone release was faster than that of high molecular growth factor and completely released within 4 weeks. The studies showed that the combination of HA with thermoreversible polymer, dexamethasone, and TGF $\beta$-3 significantly enhanced chondrogenic differentiation and expression of aggregan, collagen type I and type II.[202] Further, temperature-controlled pH-dependent chitosan solutions were prepared by addition of polyol salts bearing a single anionic head, such as

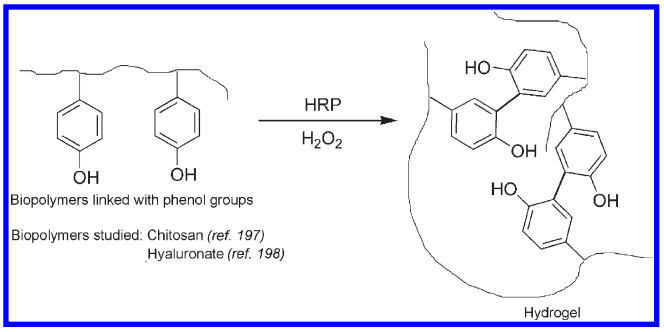


**Figure 6.** Strategy for the preparation of injectable hydrogel via enzymatic cross-linking.

glycerol−, sorbitol−, fructose−, or glucose−phosphate salts (polyol− or sugar−phosphate). Several synergistic forces are favorable to gel formation including hydrogen bonding, electrostatic interactions, and hydrophobic interactions. This set of phosphate salts gives a unique behavior by allowing the chitosan solutions to remain liquid at physiological pH and to turn into a gel upon heating to body temperature. At low temperature, strong chitosan−water interaction protects it from aggregation. Upon heating, water molecules are removed by the glycerol moiety and allow aggregation of chitosan chains. Gels loaded with bovine bone protein, an osteogenic mixture of TGF-$\beta$ family members including several BMPs and chondrocytes upon injection subcutaneously in rats, showed cartilage regeneration, demonstrating the suitability of the gels as delivery vehicles for proteins and living cells.[203] Thermosensitive chitosan prepared by grafting with poly(*N*-isopropyl acrylamide) has been used as an injectable gel for studying the differentiation of MSCs into chondrocytes. The thermosensitive gel transition was optimal for in vivo application as its LCST was 32 °C.[204] Thermosensitive injectable gels based on chitosan were also prepared by incorporating hydroxybutyl groups onto the hydroxyl and amino groups of chitosan.[205] Ibusuki and Fujii[206] attempted to develop a novel injection-driven technique for cartilage repair using a thermoresponsive gelatin, poly(*N*-isopropylacrylamide)-grafted gelatin, for cartilage tissue engineering. It has been reported that the mechanical properties of the tissue-engineered cartilage responding to loading and unloading of compression force tend to approach those of native hyaline cartilage with culture time. Temperature-triggered gels have the advantage of ease of preparation and do not require any external cross-linking agents for gelation. Major concerns of temperature-sensitive hydrogels based on *N*-isopropylacrylamide[202,204,206] are biocompatibility and biodegradability issues.[207]

### 4.5. Injectable Hydrogels via pH Change

Injectable scaffolds for cartilage engineering are reported from chitosan by making use of its gel forming property upon neutralization of aqueous solution by increasing its pH above 6.2. Injectable scaffolds based on cellulose were fabricated by selective grafting of silanol groups to hydroxypropyl methyl cellulose (Si−HPMC). Rheological analysis of Si−HPMC clearly indicates that it is initially a viscous liquid in which cells may be easily mixed and injected at the repair site. Si−HPMC solution will be of alkaline pH, while injection at high pH medium is needed for solubilzation.[208] Studies showed that the hydrogel is biocompatible and permits the growth of phenotypically stable chondrocytes, which can synthesize cartilage-like ECM. Even

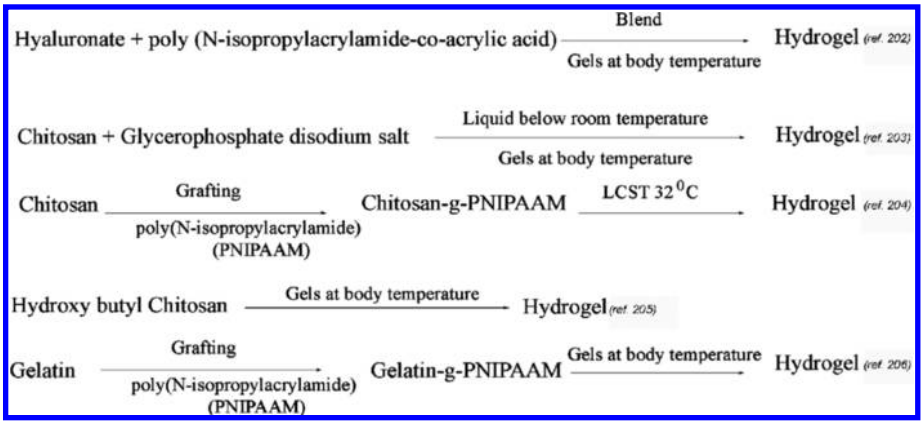


**Figure 7.** Strategy for the preparation of injectable hydrogel via temperature change.

though these systems have been evaluated for their cartilage regenerative potential, their practical use is slightly skeptical as the pH at the time of injection is either higher or lower than the physiological pH and it is difficult to predict the pH at the diseased site, which can create unwanted tissue response.

## 5. REQUIREMENTS OF THE SCAFFOLD FROM THE CLINICAL PERSPECTIVE

Many complex questions need to be answered before a scaffold is suitable for clinical evaluation. Some issues involved are as follows.

(a) Complex environment in which the construct must be evaluated in culture.
(b) Appropriate animal model predictive of human cartilage use conditions and disease state.
(c) A minimally invasive scaffold.
(d) Appropriate clinical trial design for the specific condition. This includes issues regarding concurrent pathologies, extent, severity, and duration of lesions, patient age, prior treatments, and range of motion. Evaluation parameters for pain and function assessment as well as patient follow up need to be objective and easily reproducible.
(e) Reduction in recovery period and duration of hospitalization. This can be achieved by use of the minimally invasive scaffolds as compared to conventional therapy.
(f) Appropriate degradation profiles of scaffolds. The degradation rate of the material must be known and must match those of tissue regeneration.
(g) Use of nontoxic materials which are FDA approved.

### 5.1. Animal Models

Heterotropic animal models can be used to get the foremost evidence of the chondrogenic potential of the tissue-engineered construct by implanting in dorsal subcutaneous pouches of immunocompromised nude mice. For example, primary auricular swine chondrocytes have been encapsulated in photo-cross-linkable HA hydrogels (50 $\mu$L) and implanted subcutaneously into the dorsum of nude mice. After 4 weeks the constructs resembled native cartilage with white shiny appearance. GAG content was found to increase with time and represented ~75% of those found for native articular cartilage after 12 weeks.[174] Aigner et al.[58] evaluated the efficacy of Hyaff 11 (hyaluronan benzyl ester) incorporated with humal nasal chondrocytes in a similar way and found preservation of chondrogenic potential of cells and expression of collagen type II in large areas of samples. Subcutaneous injection of in situ forming alginate hydrogels with human nasal chondrocytes (500 $\mu$L) was performed on nude mice by Dobratz et al.[209] Gels were intact at the injection site, and histological evaluation revealed 93% explants with resemblance to native cartilage after 26 weeks. Similarly, primary chondrocytes (derived from New Zealand white rabbit) mixed with RGD-modified alginate solution in the presence of $Ca^{2+}$ ions (200 $\mu$L) were injected subcutaneously into the dorsum of the nude mice. The gels were very effective in ECM generation with a large amount of sulfated GAGs as evidenced from Safranin-O staining.[186] In order to evaluate the cartilage regeneration efficacy of tissue-engineered construct as well as its bonding with host cartilage, a "rabbit pinna punch hole" model has been developed.[210] In this method four punch holes were made on the pinna of rabbit because of its ease of approach, making the procedure fast and causing minimal distress to animals. Alginate hydrogel along with demineralized bovine bone matrix has been evaluated using this model.

Subsequently, the ability of the construct to fill an articular cartilage defect is to be studied in an animal joint which anatomically, histologically, biologically, biochemically, and mechanically resembles the original joint surface. While there is no animal defect model that is directly applicable to the human, large animal models like dogs, goats, and horses closely resemble the human compared to smaller animals like rabbit. However, due to practical difficulty in carrying out experiments in large animal models, preclinical evaluation is usually conducted in small animal models.[190,211] Retention of in situ gelling chitosan solution with chondrocytes has been tested on full thickness (area, 4 × 3 $mm^2$; depth, 0.3 mm) New Zealand white rabbit joint chondral defects with unrestrained motion and load bearing for 24 h.[212]

The age of the animal also affects the cartilage repair potential and treatment response due to changes that occur in the cartilage matrix, cell, or lipid content with age.[213−217] The potential of the periosteum flap, used to anchor tissue-engineered cartilage/autologous chondrocytes to the defect, to form cartilage and repair joint surfaces is also subjected to age-related decline.[218] Therefore, proper understanding of aging-related changes and its effect on repair tissue integration will be critical to the successful reconstruction of articular cartilage by tissue-engineering techniques. The nature of the defect, including fresh (acute) or chronic defects where already some degree of synovitis and matrix degradation is present, also affects animal models. The tissue-engineered cartilage repair is fast in the case

of acute defects, whereas it is negatively influenced in chronic defects due to alterations in matrix metabolism.[219] Defect size is another parameter to be considered, and it has been proposed that creation of a "virtual" partial-thickness defect has similar dimensions to a human partial-thickness defect; however, floors and walls of the defect made impermeable to blood-borne cells and signaling substances derived from the subchondral bone-tissue spaces are more appropriate.[220] While designing the animal defect experiment models postoperative treatment after repairing the defect also should be considered. As mechanical factors influence chondrogenesis and articular cartilage healing, continuous passive motion has been experimentally found to enhance healing while joint immobilization decreases articular cartilage thickness, softens tissue, and alter PG synthesis.[221] The same is applicable for clinical use also. For comparing the different histological reports in the literature, several scoring systems have been introduced, modified, and used, out of which the scoring system by O'Driscoll and co-workers is a reliable semiquantitative cartilage scoring system with good correlation.[222]

### 5.2. Clinical Trial Design

Even though tissue engineering using scaffolds has theoretical and technical advantages over ACI in terms of minimum invasiveness, homogeneous distribution of chondrocytes, and phenotype maintenance, very few scaffold materials (collagen, fibrin, and hyaluronate based) have been marketed and clinically used. Concerns over the use of constructs with stem cells also exist as there can be uncontrolled proliferation of stem cells leading to tumorogenesis. Most of these scaffolds can be used without a periosteal or membrane coverage and can be inserted using arthroscopic techniques, making the procedure fast and less expensive. During clinical trials, magnetic resonance imaging techniques can be used for evaluation of the morphological status of the repair tissue. This will give an idea about the integration of newly formed cartilage into the surrounding tissue. The clinical outcome of commercially available tissue-engineering scaffolds reviewed by Iwasa et al.[223] shows that variable results are obtained for fibrin glue, HyalograftC, and collagen grafts depending upon the rating system used, duration of trial conducted, size and nature of the defect treated, and age of patient. Nevertheless, each appears to be as effective as conventional ACI, although none of these to date have been shown to be superior in terms of short- or mid-term clinical and histological results published. However, the tissue-engineering technique with scaffolds seems to reduce complications (septic arthritis, arthrofibrosis) or graft failures caused by the periosteal flap used in ACI.

Proper understanding of the biochemical properties and functional requirements of normal and repaired articular cartilage is vital for the progress of tissue engineering of articular cartilage. New techniques both minimally invasive and noninvasive to assess the biomechanical properties of normal, diseased, and engineered cartilage under in vivo conditions will need to be developed and standardized for the comparative evaluation of tissue-engineering techniques. Further long-term follow up is required to conclude the validity of tissue-engineering constructs as a first line of treatment of a larger cartilage defect compared with ACI.

## 6. CONCLUSIONS AND FUTURE DIRECTIONS

Clinical translation and feasibility of tissue-engineered constructs depend on several factors which include the source of cells used, the properties of the scaffolds, and the signaling molecules. The distribution of cells and growth factors in the scaffold, cues for chondrocyte regeneration from the scaffolds, the response of cells to the growth factors/signaling molecules/mechanical stimuli/nutrients, and the properties of newly formed cartilage and its integration with native tissue are the major challenges to be faced to achieve the goal.

The region (i.e., superficial or deep layers) of origin and the state of differentiation of the chondrocytes seem to control the cellular response. Long-term culture of chondrocytes derived from young donors with high initial cell seeding density shows good results with respect to growth rate, in vitro expansion, and biosynthesis. The use of mechanical stimuli and growth factors appears to be quite promising to promote differentiation and biosynthesis of chondrocytes.

Key issues related to scaffolds are their biocompatibility, biodegradability, porosity, mechanical stability, and delivery to site of injury. Hydrogels derived from biopolymers have synergistic beneficial aspects of water swelling, cartilage-like behavior of hydrogel, as well as extracellular matrix mimicking property of biopolymers. The poor mechanical stability of these matrices and nonadhesive nature of cells onto the surface have been rectified to some extent by the hybridization of biopolymers with synthetic polymers and incorporation of peptides. In situ gelling, injectable scaffolds offer many advantages over the preformed matrices, but preparation of those systems will require some toxic cross-linking agents, or sometimes the gelling will not be rapid enough to stay at the site of injury or the resulting temperature will be too high to cause harm to nearby tissues. Therefore, injectable systems should be developed by taking all these major issues into consideration without compromising the beneficial aspects of biopolymers. Much attention should be given to develop injectable hybrid systems as those systems can solve the issues related with targeted minimal invasive delivery, biocompatibility, and mechanical strength.

To date, most of the studies have been short term and conducted on animal models using animal cells. Very few works are reported on studies based on human chondrocytes/mesenchymal cells seeded onto hydrogels for cartilage tissue engineering. The focus of all these studies are on the rates of the biosynthesis of matrix molecules such as proteoglycan and collagen and the macroscopic mechanical properties of the engineered constructs. The long-term outcome will be clearer if the information regarding the organization of matrix molecules and cross-linking of the collagen and proteoglycan network is obtained. Nothing will be equal to the human body system; therefore, the most efficient tissue-engineering strategy possibly will be the use of in vitro culture only to direct the initial development of the construct with further development taking place in vivo. The ultimate success of any tissue-engineering procedure is functional integration of the engineered tissue with the patient's tissue at the injury site which can only be determined by long-term in vivo studies.

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## BIOGRAPHIES

Dr. Biji Balakrishnan received her B.Sc. degree in Chemistry from Calicut University and Master's degree in Applied Chemistry from Cochin University of Science & Technology, Kerala, India, with highest honors in 1997 and 1999, respectively. She received her Ph.D. degree in Biomaterials from Sree Chitra Tirunal Institute for Medical Sciences & Technology, Kerala, India, in 2006 under the guidance of Dr. A. Jayakrishnan. Her dissertation was focused on development of in situ gelling systems based on biopolymers for various biomedical applications. She received the Shah-Schulman award for the best Ph.D. thesis in the area of colloids and interfacial science in 2007, which is an award in line with the V. K. Lamar Award for the best Ph.D. Thesis in Surface and Colloid Science instituted by the American Chemical Society in 1960. She joined the research group of Professor Rinti Banerjee, Department of Biosciences & Bioengineering, IIT, Bombay, India, in 2006 as a Postdoctoral Fellow. Currently, she is working on a project funded by the Department of Science & Technology, Government of India, on the development of biopolymer-based injectable scaffolds for cartilage tissue engineering. She has 8 publications to her credit in peer-reviewed international journals.

Dr. Rinti Banerjee is a Professor of Biomedical Engineering at the Department of Biosciences & Bioengineering and the Centre for Research in Nanotechnology and Science and WRCBB at the Indian Institute of Technology, Bombay, India. She received her MBBS degree from BJ Medical College Pune and Ph.D. in Biomedical Engineering from IIT Bombay. She did postdoctoral work at the University of California, San Francisco, before joining IIT Bombay as a faculty in 2001. Her research interests include nanomedicine, pulmonary surfactant replacements, lipid and biopolymeric materials for drug delivery, and tissue engineering. She has published over 90 papers in these areas including invited editorials and books. She has received several awards in recognition of her work including the Indo-US Frontiers of Engineering Award 2010, Annual Felicitation Award from the Society for Cancer Research and Communications in 2008, several young scientist awards from the International Foundation of Science, Sweden, DAE, India, DST, India, and AICTE Career Award for Young Teachers. She is a member of the Editorial Board of several international journals like *Drug Delivery and Translational Research* and *Biomaterials and Biodevices* and is an Associate Editor of the *Journal of Biomaterials and Tissue Engineering*. Many of the technologies developed by Dr. Banerjee have been filed as patents and are being evaluated for clinical translation.

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## ABBREVIATIONS

| | |
|---|---|
| PDGF | platelet derived growth factor |
| FGF | fibroblast growth factor |
| IGF | insulin-like growth factor |
| TGF | transforming growth factor |
| BMP | bone morphogenic protein |
| PG | proteoglycan |
| ECM | extracellular matrix |
| GAG | glycosaminoglycan |
| HA | hyaluronic acid |
| IKDC | International Knee Documentation Committee |
| CS | chondroitin sulfate |
| RGD | arginine−glycine−aspartic acid sequence |
| RGE | arginine−glycine−glutamic acid sequence |
| BMMSC | bone marrow mesenchymal stem cell |
| MSC | mesenchymal stem cell |
| ACI | autologous chondrocyte implantation |
| PLGA | poly(lactic-*co*-glycolic) acid |
| GCH | gelatin/chondroitin/hyaluronate |
| PEG | poly(ethylene glycol) |
| PVA | poly(vinyl alcohol) |
| GHC6S | gelatin/hyaluronicacid/chondroitin-6-sulfate |
| MA | methacrylate |
| PEGDM | poly(ethylene glycol dimethacrylate) |
| PEODA | poly(ethylene oxide diacrylate) |
| AEMA | 2-aminoethyl methacrylate |
| HRP | horseradish peroxidase |

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