16 Natural Polymers in Tissue Engineering Applications

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OUTLINE

Objectives

- To understand the origin, structure, and properties of natural polymers used in tissue engineering (TE) applications
- To identify the characteristics that make natural polymers interesting for TE applications
- To understand the possible factors that may affect cells/tissue response to natural polymer-based scaffolds
- To understand the possible specific applications of each natural polymer in the context of tissue engineering
- To understand the processing possibilities of the different natural origin polymers for TE applications
- To recognize the most important achievements in this research field attained by different scientists
- To understand the versatility obtained by combining natural origin polymers with other materials in TE applications

Perhaps appropriately designed biodegradable templates can be used to regenerate segments of other tissues or organs which have become lost or dysfunctional due to disease or trauma [313].

Yannas et al (1982)

16.1 Introduction

Life as we know it could not exist without natural polymers. Just think of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). They are natural polymers essential in many life processes. In fact, long before there were plastics and synthetic polymers, nature was using natural polymers to make life possible. In the early 1900s, scientists began to understand the chemical makeup of natural polymers and how to make synthetic polymers with properties that complement those of natural materials. Nevertheless, for many purposes, we still do not think of natural polymers in the same way as we think about synthetic polymers. However, that does not make natural polymers less important; indeed, it turns out that they are more important in many ways. In fact,

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after a century of developing synthetic polymers for use as materials, polymer science is turning back toward its roots, as natural polymers show promise in a wide range of biomedical uses, such as scaffolds for growing artificial human tissues, that is, for making life better after injury or disease.

Tissue engineering offers the possibility to help in the regeneration of tissues damaged by disease or trauma and, in some cases, to create new tissues and replace failing or malfunctioning organs. Typically, this is achieved through the use of degradable biomaterials to either induce surrounding tissue and cell ingrowth or to serve as temporary scaffolds for transplanted cells to attach, grow, and maintain differentiated functions. In any case, the role of the biomaterial scaffold is temporary, but still crucial to the success of the strategy. Therefore, the design and production of an appropriate scaffold material is the first, and one of the most important stages, in tissue engineering strategies. In this critical stage, the selection of the most adequate raw material is a primary consideration. Natural polymers were the first to be used as scaffold materials for tissue regeneration. They have frequently been used in tissue engineering applications because they are either components of, or have properties similar to, the natural extracellular matrix (ECM).

This chapter provides an overview of the natural origin polymers that are commercially available or currently being studied in different labs for tissue engineering applications, with some emphasis on the most widely studied systems. It describes their chemical structure, main properties, and potential applications within the field. Several aspects regarding the development and research status toward their final application are addressed. The main advantages and disadvantages of the use of natural origin polymers as compared to other materials used in tissue engineering scaffolding are also discussed.

16.2 Natural Polymers

Natural polymers are derived from renewable resources such as plants, animals, and microorganisms, and are, therefore, widely distributed in nature. These materials exhibit a large diversity of unique (and in most cases) rather complex structures, and different physiological functions, and may offer a variety of potential applications in the field of tissue engineering due to their various properties, such as pseudoplastic behavior, gelation ability, water-binding capacity, and biodegradability, among many others. In addition, they possess many functional groups (amino, carboxylic, and hydroxyl groups) available for chemical (hydrolysis, oxidation, reduction, esterification, etherification, cross-linking reactions, etc.) [1,2] and enzymatic [3,4] modification and/or conjugation with other molecules, which allows an overwhelming variety of products with tailorable chemistries and properties to be obtained. Protein materials may offer an additional advantage as they are able to interact favorably with cells through specific recognition domains present in their structure. On the other hand, the creation of hybrid materials—by means of combining the advantages of different natural polymers—may constitute a useful approach to mimicking the natural environment of the ECM and to obtaining scaffolding materials with superior mechanical and biological properties.

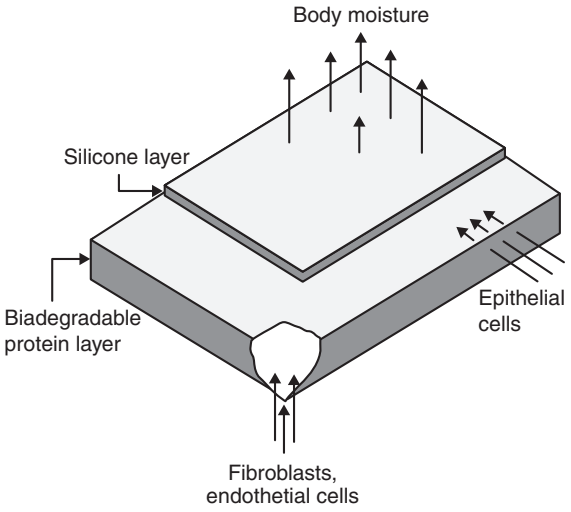
An intrinsic characteristic of natural origin polymers is their ability to be degraded by naturally occurring enzymes, which may indicate the greater propensity of these materials to be metabolized by the physiological mechanisms. Another important aspect to consider when using natural polymers, is that they can induce an undesirable immune response due to the presence of impurities and endotoxins (depending on their source), and their properties may differ from batch to batch during large-scale isolation procedures due to the inability to accurately control the processing techniques. Nevertheless, as knowledge about these natural polymers increases, new approaches (including methods for production, purification, controlling material properties, and enhancing material biocompatibility) are likely to be developed for designing better scaffolding materials to support the development of more natural and functional tissues.

In summary, both natural and synthetic polymers present important characteristics and, therefore, one must recognize that the best biodegradable polymer for biomedical applications might be found by taking steps toward the development of new biomaterials that combine the most favorable properties of synthetic and natural polymers. Several examples of the combination of natural and synthetic polymers will be described in further sections. Another approach that will be presented consists of the reinforcement of polymeric matrices with bioactive ceramic materials, such as hydroxyapatite and other calcium phosphates. These fillers have the ability, in most cases, to improve the mechanical properties and the biological behavior simultaneously.

It is well known that living organisms are able to synthesize a vast variety of polymers that can be divided into eight major classes according to their chemical structure: (1) polysaccharides, (2) proteins and other polyamides, (3) polyoxoesters (polyhydroxyalkanoic acids), (4) polythioesters, (5) polyanhydrides (polyphosphate), (6) polyisoprenoids, (7) lignin, and (8) nucleic acids [5]. However, only polymers belonging to the three first classes will be described in more detail in this chapter. This is due to their importance as raw materials in tissue engineering scaffolding. Although most of these natural polymers are obtained from plant [6–8] and animal [9,10] sources or from algae [11], there are a large number of microorganisms capable of synthesizing many biopolymers. In fact, with advances in biotechnology, there is an increasing interest in using microorganisms to produce polymers by fermentation (enabling large-scale production, avoiding complex and time-consuming isolation procedures, and the risk of animal-derived pathogens) [12,13] or *in vitro* enzymatic processes [14]. This makes it possible to control polymer molecular weight, branching patterns and branch chain lengths, and cross-linking between chains, altering the fine structure, and functional properties of polymers.

16.2.1 Classical Experiment

While the term *tissue engineering* was still to be "coined" (this happened only in 1987), researchers were already studying an approach to regenerate skin wounds, which resulted in a paper published in *Science* in 1982 [15]. This paper described the prompt and long-term closure of full-thickness skin wounds in guinea pigs and humans, achieved by applying a bilayer polymeric membrane, comprising of a top silicone layer and a bottom layer of a porous cross-linked network of collagen and glycosaminoglycan (GAG), seeded with a small number of autologous basal cells before grafting [15]. This study was conducted following three main stages, today recognized by many researchers as the three main phases of tissue engineering approach: The first stage corresponds to the development of the material matrix membranes; in the second stage, the membranes are seeded with cells; and finally, in the third stage, the cell seeded membranes are grafted onto the tissue defect. The first stage, i.e., the development of the appropriate membranes based on collagen and GAG, was in fact, extensively explored, as the authors have analyzed a range of different chemical compositions and several methods were compared for preparing membranes with different porosities and pore sizes [16–18]. Extensive characterization of the materials led to the selection of the most suitable formulation/structure to be used in the further stages of the development of these skin tissue equivalents. Neodermal tissue synthesis occurred in these membranes seeded with autologous cells and there was no evidence of conventional scar formation. New and apparently normal functional skin was generated in less than 4 weeks. It was demonstrated that, although the acellular membranes can also be used to regenerate skin defects, the cell-seeded membranes provide a means for closing the largest full-thickness skin wounds in a shorter period of time [15]. This system, mainly based on a 3D polymeric matrix obtained from two natural origin polymers (collagen and GAG), also resulted in the first tissue-engineered product to be approved by FDA (1996), and clearly opened the way to the concept of tissue engineering, i.e., to the regeneration of tissues using cells seeded onto a 3D matrix made from the natural-origin polymers.

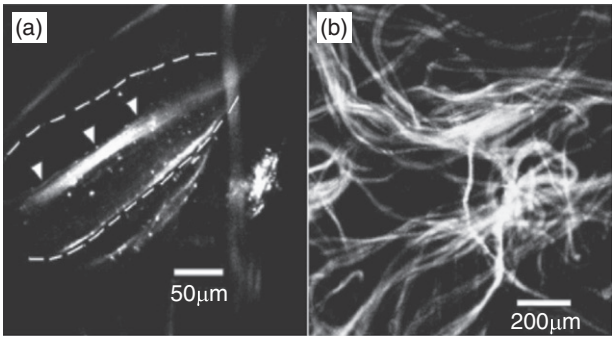


Schematic representation of the polymeric membrane developed by Yannas et al. [15] (*Wound tissue can utilize a polymeric template to synthesize a functional extension of skin.* Science, *215*(*4529*): 174–176) *to be used as a template to obtain skin substitutes. The top layer, made of medical grade silicone, is designed to be spontaneously ejected following formation of a confluent neoepidermal layer under it. The bottom layer, a cross-linked network of collagen and chondroitin 6-sulfate, was designed to undergo biodegradation at a controlled rate while it is replaced by neodermal tissue.*

16.2.2 State of the Art Experiment

16.2.2.1 Natural Polymers in Gene Delivery and Tissue Engineering

Research on natural polymers for the development of matrix-based gene delivery systems has opened the way to new and exciting possibilities to be explored within the field of regenerative medicine [19]. In fact, the combination of gene therapy and tissue engineering exploits the potential of genetic cell engineering to provide biochemical signals that direct cell proliferation and differentiation, and simultaneously, the ability of natural polymers to serve as gene carriers and tissue engineering scaffolds. It is true that synthetic polymers and viral carriers have been preferentially used in gene delivery applications, but natural polymers have unique and intrinsic properties that can make them more suitable candidates for this type of application. Such properties include their general biocompatibility, mucoadhesive character, and biodegradability. The biocompatibility of natural polymers, e.g., allows for cells infiltration into the matrix and transfection can occur as these cells come into contact with the imbedded DNA. The biodegradability of the matrices obtained from natural polymers may also assist the release of gene transfer agents into the surrounding environment and thus affect nearby cells. The current research suggests therefore, that natural polymeric carriers have a different mechanism for intracellular escape and transfection than synthetic polymers. However, a very limited number of studies have focused on the development of matrices based on natural polymers for gene delivery and for cell support. An interesting example is provided by research work from Lim *et al.* [20] in which the authors have investigated a 3D fiber-mesh scaffold, based on chitin and alginate, as a way to obtain a better spatial control of plasmid localization, in opposition to other available systems that are based on simple mixture to bond the matrix and the gene delivery elements. In this study, chitin and alginate fibers were formed by polyelectrolyte complexation of the water-soluble polymers, and PEI–DNA nanoparticles containing green fluorescent protein (GFP)-encoding plasmid were loaded during the fiber drawing process. These fibers were then processed into a nonwoven fiber-mesh scaffold, using a method based on the needle-punching technique. This system was then studied to analyze the tranfectability of human epithelial kidney (HEK293) cells and human dermal fibroblasts (HDFs) seeded on the scaffolds. In summary, the results obtained showed that nanoparticles released from the fibers over time retained their bioactivity and successfully transfected cells seeded on the scaffold in a sustained manner. Transgene expression in HEK293 cells and HDFs seeded on the transfecting scaffolds was significant even after 2 weeks of culture compared to 3-day expression in 2D controls. Fibroblasts seeded on scaffolds containing DNA encoding basic fibroblast growth factor (bFGF) demonstrated prolonged secretion of bFGF at levels significantly higher than baseline.



Confocal microscopy images of the fibrous scaffolds and the PEI–DNA nanoparticles (fluorescently stained) encapsulated within the fibers developed by Lim et al. *(Nonviral gene delivery from nonwoven fibrous scaffolds fabricated by interfacial complexation of polyelectrolytes.* Mol Ther, *13(6): 1163–1172, 2006). (a) Phase microscopy showing the bead region of a single fiber, depicting the nanoparticles dispersed within the bead (dotted lines) and at a higher density in the core fiber segment (arrows); (b) fibers containing nanoparticles.*

16.3 Polysaccharides

Polysaccharides, also known as glycans, consist of monosaccharides (aldoses or ketoses) linked together by *O*-glycosidic linkages. Each monosaccharide is classified according to the number of carbons in the monosaccharide chain (usually 3–9), into trioses (C3), tetroses (C4), pentoses (C5), hexoses (C6), heptoses (C7), octoses (C8), and nonoses (C9). Polysaccharides can be classified as homopolysaccharides or heteropolysaccharides if they consist of one type or more than one type of monosaccharide. Because glycosidic linkages can be made to any of the hydroxyl groups of a monosaccharide, polysaccharides form linear as well

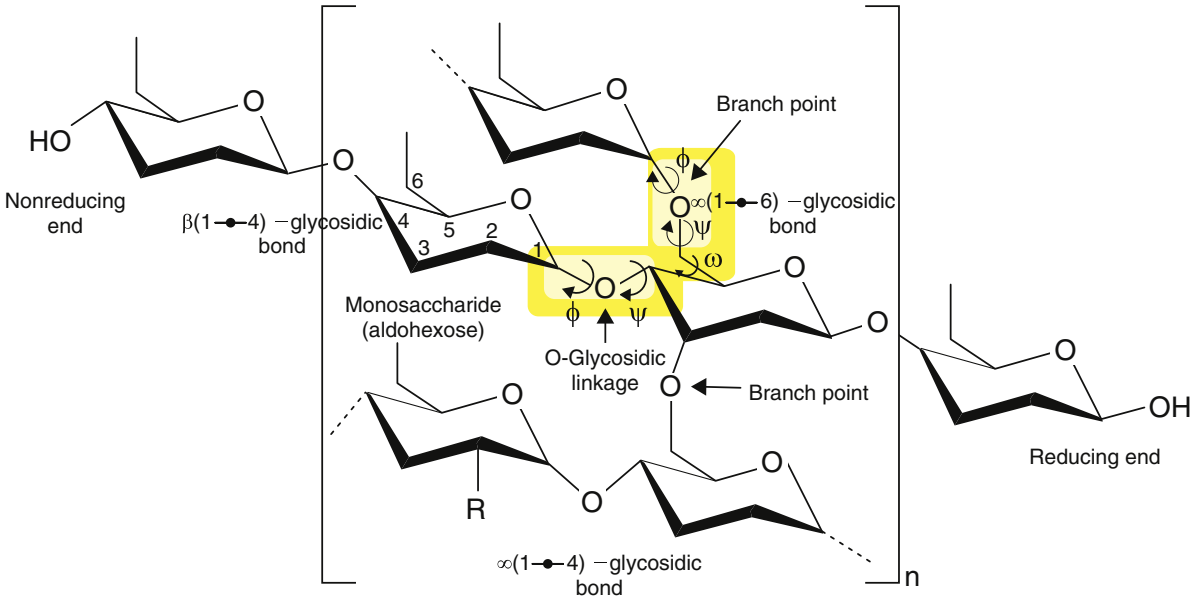


Figure 16.1 General structure of polysaccharides showing their diversity in terms of monosaccharide composition (nature and molar ratios of the monosaccharide building blocks), linkage patterns (linkage positions between the glycosidic linkages and branches), anomeric configuration (α- or β-configuration of the glycosidic linkage), substitutions (position and nature of OH_2 modifications), degree of freedom in (1→4) and (1→6)-glycosidic bonds. *Source: Izydorczyk, M. (2005).* Understand the Chemistry of Food Carbohydrates. Food Carbohydrates: Chemistry, Physical Properties, and Application *(Cui, S.W. ed.), Boca Raton, CRC Press, Taylor & Francis Group: 1–65.*

as branched polymers (Fig. 16.1). Differences in the monosaccharide composition, linkage types and patterns, chain shapes, and molecular weight, dictates their physical properties, including solubility, flow behavior, gelling potential, and/or surface and interfacial properties [9,21].

In the living organisms, polysaccharides perform a range of biological functions, such as maintenance and structural integrity (e.g., cellulose, chitin), energy reserve storage (e.g., starch, glycogen), and biological protection and adhesion (e.g., gum exudates, extracellular microbial polysaccharides). These functions can be found in the compilation presented in Table 16.1.

In this chapter, the authors have chosen to focus only on the polymers that have been proposed, by different researchers, for application within the tissue engineering field; namely alginate, dextran, chitosan, cellulose, starch, and hyaluronic acid (HA) polysaccharides. All of these polymers have been used as scaffold materials and will be described in more detail in the following sections.

16.3.1 Alginate and Dextran

Alginate is a biological material derived from sea algae, composed of linear block copolymers of 1–4 linked β-D-mannuronic acid (*M*) and α-L-guluronic acid (*G*) (Fig. 16.2). Divalent ions form cross-links in alginate by binding the guluronic residues, inducing a sol–gel transition in the material.

Dextran is a bacterial-derived polysaccharide, consisting essentially of α-1,6 linked D-glucopyranose residues with a few percentage of α-1,2-, α-1,3-, or α-1,4-linked side chains (Fig. 16.3) synthesized from sucrose by *Leuconostoc mesenteroides* and *Streptococcus* sp.

Because of their biocompatibility, abundance in source, and low prices, they have been widely used in the food industry as thickeners and emulsifying agents. Alginate can be ionically cross-linked by the addition of divalent cations (like Ca^{2+}) in aqueous solution. The gelation and cross-linking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations, and the stacking of these guluronic groups to form the characteristic egg-box structure shown in Fig. 16.4.

The cross-links are believed to create a stiff egg-box structure and they impart viscoelastic solid behavior to the material [22,23]. The properties of alginate derive from this behavior, and include [24–26] a relatively inert aqueous environment within the matrix; a high gel porosity that allows for

Table 16.1 Classification of Polysaccharides According with Their Origin, Function, Linkage Patterns, Sequence and Composition of Sugar Units in Polysaccharide Chains, and Presence of Ionizing Groups

Origin	Polysaccharide	Occurrence/Function	Glycosidic Linkage/ Repeating Unit	Nature and Distribution of the Monosaccharide Units
Plant	Starch	Starch is synthesized in amyloplasts of green in plants and deposited in the major depots of seeds, tubers, and roots in the form of granules. Energy storage material in almost higher plants (corn, rice, potato, wheat, tapioca, etc).	Amylose: α-(1→4)-D-Glc Amylopectin: α-(1→4, 1→6)-D-Glc	Homopolysaccharide: neutral Amylose: linear Amylopectin: branched
	Cellulose	Structural polysaccharide in the cell walls of higher plants (cotton, wood). Besides the mechanical strength of the plant cell, cellulose is a protective component against external attack by mechanical forces or microorganisms.	β-(1→4)-D-Glc	Homopolysaccharide: neutral, linear
	Arabinogalactan	Larch arabinogalactan is extracted from the heartwood of the western larch *Larix occidentalis*. It is an exudate gum polysaccharide that is produced on the exterior surfaces of the plant usually as a result of trauma or stress (physical injury and/or fungal attack).	Main chain: β-(1→3)-D-Gal Side chains: disaccharides β-D-Gal-(1→6)-β-D-Gal and β-L-Ara-(1→3)-α,-L-Ara	Heteropolysaccharide: neutral, branched
Algal	Alginate	It occurs combined with calcium and other bases in the cell walls and intracellular matrix of brown seaweeds (*Phaeophyceae*), being the main structural component. Contributes to ionic interactions and physical protection.	β-(1→4)-D-ManA-α(1→4)-L-GulA	Heteropolysaccharide: anionic, linear
	Agarose	Red algae (*Rhodophyceae*). Biological function in algae is antidessication at low tide and to provide mechanical support so that cells do not collapse.	-(1→3)-β-D-Gal-(1→4)-3,6-anhydro-α-L-Gal	Heteropolysaccharide: neutral, linear
	Carrageenans	Carrageenans are structural polysaccharides of the marine red algae (*Rhodophyceae*).	j-carrageenan: -(1→3)-β-D-Gal-4-sulfate-(1→]4)-3,6-anhydro-α-D-Gal-(1→3)-	Heteropolysaccharide: anionic, linear

(*Continued*)

Table 16.1 Classification of Polysaccharides According with Their Origin, Function, Linkage Patterns, Sequence and Composition of Sugar Units in Polysaccharide Chains, and Presence of Ionizing Groups—Cont'd

Origin	Polysaccharide	Occurrence/Function	Glycosidic Linkage/ Repeating Unit	Nature and Distribution of the Monosaccharide Units
			κ-carrageenan(1→3)-β-D-Gal-4-sulfate-(1→4)-3,6-anhydro-a-D-Gal-2-sulfate-(1→3)- k-carrageenan: (1→3)-β-D-Gal-2-sulfate-(1→4)–α-D-Gal-2,6-disulfate-(1→3)-	
Animal	Chitin/chitosan	Chitin is the main component of the exoskeleton of insects and shells of crustaceans (crab, shrimp, lobster, etc.). Structural/ supporting polysaccharide. Chitosan is a chitin derivative obtained by a deacetylation reaction.	Chitin: 1-(1→4)-D-GlcNAc Chitosan: β-(1→4)-D-GlcN-α-(1→4)-D-GlcNAc, distributed in a random way depending on the degree of acetylation.	Chitin: homopolysaccharide, neutral, linear Chitosan: heteropolysaccharide, cationic, linear
	Hyaluronic acid	Hyaluronan is an important glycosaminoglycan component of connective tissue (cartilage, tendon, skin, and blood vessel walls), synovial fluid (the fluid that lubricates joints) and the vitreous humor of the eye. It plays a significant role in wound healing.	-β(1→4)-D-GlcUA-β(1→3)-D-GlcNAc-	Heteropolysaccharide: anionic, linear
Microbial	Dextran	Extracellular polysaccharide produced by the bacterium *Leuconostoc mesenteroides.*	α-(1→2, 1→3, 1→4, 1→6)-Glc	Homopolysaccharide: neutral, branched
	Gellan gum	Extracellular polysaccharide produced by the bacterium *Sphingomonas elodea.*	→3)-β-D-Glc-(1→4)-b-D-GlcUA- (1→4)-β-D-Glc-(1→4)-α-L-Rha-(1→	Heteropolysaccharide, anionic, linear
	Pullulan	Extracellular polysaccharide produced by the fungus *Aureobasidium pullulans*.	α-(1→6)-maltotriose	Homopolysaccharide: neutral, branched

Glc, glucose; Ara, arabinose; GulA, guluronic acid; ManA, mannuronic acid; Gal, galactose; GlcNAc, N-*acetylglucosamine; GlcN,* N-*glucosamine; GlcUA, glucuronic acid, Rha, rhamnose*

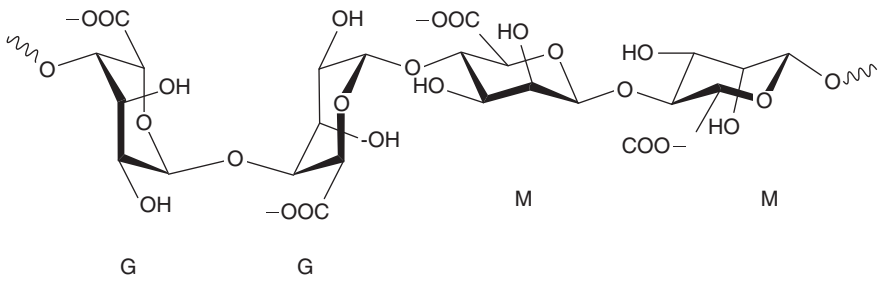


Figure 16.2 Alginate structure. M − mannuronic acid; G − guluronic acid.

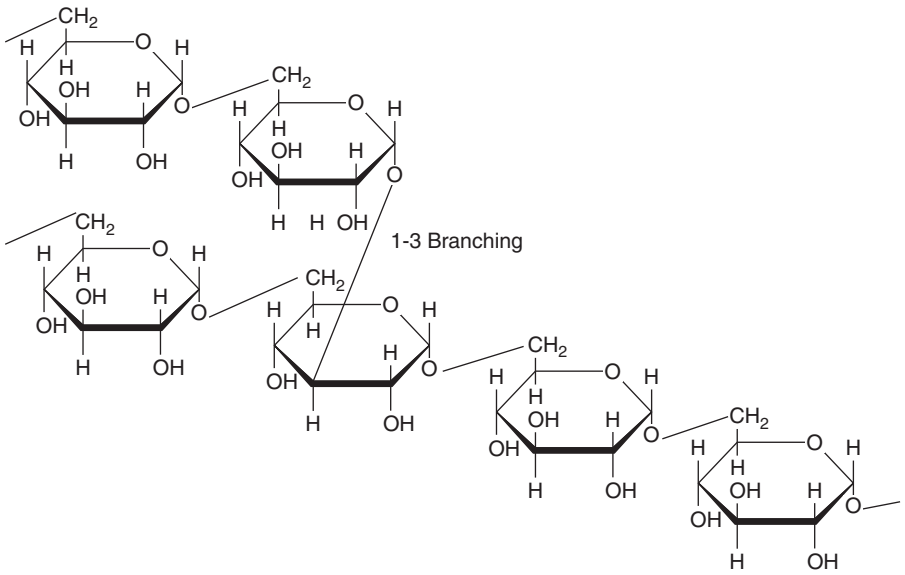


Figure 16.3 Dextran structure evidencing the 1−3 branching.

high diffusion rates of macromolecules; the ability to control this porosity with simple coating procedures and dissolution and biodegradation of the systems under normal physiological conditions and, at room temperature, a mild encapsulation process free of organic solvents. An attractive class of physically cross-linked gels are those where gel formation is not instantaneous, but occurs a certain time after mixing the hydrogel components or after a certain trigger (such as pH or temperature). These systems can be administered by injection as liquid formulation and gellify *in situ* [27−29].

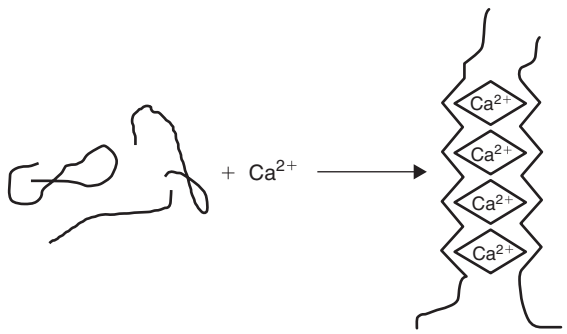


Figure 16.4 Egg-box model for alginate gel formation. It is shown the conversion of random coils to buckled ribbon-like structures containing arrays of Ca^{2+} ions (e).

It is the latter characteristic that drew the attention of researchers to use alginate for encapsulation of cells as well as bioactive agents. The material to be encapsulated is usually mixed with an alginate solution, and the mixture dripped into a solution containing Ca^{2+} ions, resulting in the instantaneous formation of microparticles that entrap cells or drugs within a 3D lattice [30]. Dextran hydrogels can be created by either physical or chemical cross-linking, taking advantage of the hydroxyl groups present on the α-1,6-linked D-glucose residues. Dextran particles have been widely used as separation matrices, such as Sephadex, as cell microcarriers, such as Cytodex, and as drug delivery vehicles [31]. There has been considerable interest on dextran scaffolds for tissue engineering applications [31,32].

Similarly, alginate cross-linked with Ca^{2+} has been popularized for *in vitro* cell culture [33,34] and tissue engineering applications [27,35−39] primarily because of the ability to immobilize and later recover cells from the culture matrix [23]. Alginate has also been used as a bioartificial matrix for cartilage

generation and fundamental studies on entrapped chondrocytes [33,39]. The suspension of cells in a bioartificial matrix, such as alginate, is associated with significant changes in the local physical and mechanical environment of the cells compared to their native ECM [23]. ECM plays an important role in tissue engineering because cellular growth and differentiation, in the 2D cell culture as well as in the 3D space of the developing organism, require ECM with which the cells can interact [40]. In the artificial culture system, the physical properties of the artificial matrix will govern the deformations and tractions applied to the cells, altering important cell−matrix interactions present in the native system that appear to regulate cell activity in response to mechanical stress [33,41]. On the other hand, alginate is well known for forming strong complexes with polycations including, but not limited to, synthetic polymers, proteins, and polypeptides. This feature is particularly important when attempting to use alginate as a scaffold for tissue engineering applications such as cartilage and bone, since mechanical constraints limit its applications. Combining alginate with other polymers and ceramic materials has been shown to be able to obviate this feature [42−46].

Alginate has also been widely studied for engineering liver tissue [47−49]. The bioartificial liver-assist device or regeneration of the liver-tissue substitutes for liver tissue engineering requires a suitable ECM for hepatocyte culture because hepatocytes are anchorage-dependent cells and are highly sensitive to the ECM milieu for the maintenance of their viability and differentiated functions [40]. A potential approach to facilitate the performance of implanted hepatocytes is to enable their aggregation and reexpression of their differentiated function prior to implantation [49] and alginate has been shown to allow hepatocyte culture and function.

The differentiation and growth of adult stem cells within engineered tissue constructs are believed to be under the influence of cell-biomaterial interactions. Gimble et al. [50] have shown that alginate-based materials can have an enhancing effect over the differentiation of human adipose-derived adult stem (hADAS) cells, and manipulating the composition of these tissue engineered constructs may have significant effects on their mechanical properties. Additionally, the major role of alginate in tissue engineering has been defined as a vehicle for cell encapsulation and delivery to the site, and attachment of RGD sequences has shown to potentate bone cell attachment and upregulation of specific bone markers [51].

In summary, the possibility of having an injectable *in situ*-gellifying material that can serve as a filler and template for the regeneration/repair of tissues such as cartilage, is a very attractive one. Alginate and dextran have shown and continue to show excellent properties for this purpose. Allied to this, the potential of being tailored for several applications—hence a multitasking ability for these materials—renders interest from the scientific community.

16.3.2 Chitosan

During the past 30 years, a substantial amount of work has been published on chitosan and its potential use in various pharmaceutical applications [52], including tissue engineering. This is due to its similar structure to naturally occurring GAG and its degradability by enzymes in humans [53]. Figure 16.5 shows a chitosan structure. It is a linear polysaccharide of (1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine residues derived from chitin, a high molecular weight, the second most abundant natural biopolymer commonly found in arthropod exoskeletons such as shells of marine crustaceans and cell walls of fungi [54]. Chitosan has been proven to be biologically renewable, biodegradable [55−57], bioadhesive [58,59], and biocompatible [52,55,60−63], and used in wound dressing and healing [55,64,65], drug delivery systems [66−69], and various tissue engineering applications. We focus on these issues in this chapter.

Depending on the source and preparation procedure, chitosan's average molecular weight may range from 50 to 1000 kDa [70]. The degree of *N*-deacetylation usually varies from 50 to 90% [52]. Chitosan is a semicrystalline polymer and the degree

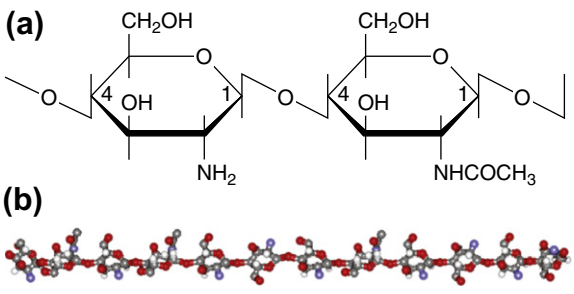


Figure 16.5 Structure of chitosan.

of crystallinity is a function of the degree of deacetylation. Crystallinity is maximum for both chitin (i.e., 0% deacetylated) and fully deacetylated (i.e., 100%) chitosan. Minimum crystallinity is achieved at intermediate degrees of deacetylation. Chitosan is degraded by lysozyme [18,57,71]; the kinetics of degradation is inversely related to the degree of crystallinity. Because of the stable crystalline structure, chitosan is normally insoluble in aqueous solutions above pH 7. However, in dilute acids, the free amino groups are protonated and the molecule becomes fully soluble below pH 5. The pH-dependent solubility of chitosan provides a convenient mechanism for processing under mild conditions. Viscous solutions can be extruded and gelled in high pH solutions [72] or baths of nonsolvents such as methanol. Such gel forms (particles, fibers, or blocks) can be subsequently drawn and dried to form high-strength materials. Much of the potential of chitosan as a biomaterial stems from its cationic nature and high charge density in solution. The charge density allows chitosan to form insoluble ionic complexes or complex coacervates with a wide variety of water-soluble anionic polymers. Chitosan derivatives and blends have also been gelled via glutaraldehyde cross-linking [73] and other cross-linking agents such as genipin [74], UV irradiation [53], and thermal variations [54]. Besides the referred gelling-based processing method, freeze-drying is undoubtedly the most widely processing technology used to process chitosan shapes. Furthermore, the cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic GAGs, proteoglycans, and other negatively charged molecules. This property is of great interest because a large number of cytokines/growth factors are linked to GAG, and a scaffold incorporating a chitosan−GAG complex may retain and concentrate growth factors secreted by colonizing cells [54]. Several researchers have examined the host tissue response to chitosan-based materials. In general, these materials evoke a minimal foreign body reaction, with little or no fibrous encapsulation [75]. Formation of normal granulation tissue associated with accelerated angiogenesis, appears to be the typical course of the healing response. This immunomodulatory effect has been suggested to stimulate the integration of the implanted material by the host [76].

Due to its promising properties, chitosan has been applied in tissue engineering applications targeting several tissues and these are summarized in Table 16.2. The most commonly aimed tissues are bone, cartilage, and skin, but others such as liver or trachea have applied chitosan as scaffolds to support the temporary cell functions. Due to its easy processability, chitosan has been molded in a range of shapes including porous scaffolds, injectable gels, membranes, tubular systems, and particles as described in Table 16.2. Chitosan scaffolds for bone tissue engineering have been widely investigated and shown to enhance bone formation both *in vitro* and *in vivo*, mainly in the presence of other polymers such as gelatin [73] and alginates [44]. When one considers cartilage tissue engineering applications, in particular, chitosan seems to be a good candidate given the importance of GAGs in stimulating the chondrogenesis [77], the use of GAGs or GAG analogs such as chitosan as components of a cartilage tissue scaffold appears to be a logical approach for enhancing chondrogenesis as shown by several papers [76−79]. It thus shares some characteristics with various GAGs and HA present in articular cartilage [70].

At present, chitosan is one of the most promising natural origin polymers for tissue engineering. In particular, its chemical versatility and the possibility to generate structures with predictable pore sizes and degradation rates make chitosan a promising candidate scaffold for these applications. In fact, the combination of good biocompatibility, intrinsic antibacterial activity, and ability to bind to growth factors renders this material as a good potential for several tissue engineering applications.

16.3.3 Cellulose

Cellulose is the main component of plant cell walls. It also constitutes the most abundant, renewable polymer resource available today, existing mainly in lignocellulosic material in forests, with wood being the most important source. The primary structure of this linear polymer consists of up to 15,000 D-glucose residues linked by β(1→4)-glycosidic bond [80] (Fig. 16.6). The fully equatorial conformation of β-linked glucopyranose residues stabilizes the chain structure, minimizing its flexibility. It is the ability of these chains to hydrogen-bond together into fibers (microfibrils) that give cellulose its unique properties of mechanical strength and chemical stability, leading also to insoluble materials with small degradability *in vivo* [81].

Table 16.2 Chitosan-Based Scaffolds for Different Tissue Engineering Applications

Material	Scaffold Structure	Processing Methodology	Cell type (Source)	TE Application	References
Chitosan	3D fiber meshes	Wet spinning	Osteoblast-like SAOS-2 (human osteosarcoma cell line)	Bone	[271]
Chitosan	3D porous blocks	Freeze-drying	Osteoblast-like ROS (rat osteosarcoma cell line)	Bone	[272,273]
Chitosan/ polyester	3D fiber meshes	Fiber extrusion	MSCs (human bone marrow, primary culture)	Bone	[274–276]
Chitosan/ alginate	3D porous cylinders	Freeze-drying	Osteoblast-like MG63 (human osteosarcoma cell line)	Bone	[44]
Chitosan/ alginate	Injectable gel	Gelation by sonication	MSCs (rat bone marrow, primary culture)	Bone	[277]
Chitosan/ (HA)	3D porous cylinders	Particle aggregation	ADAS cells (human adipose tissue, primary culture)	Bone	[72]
Chitosan/ (HA)	3D porous cuboids	3D-printing	Osteoblasts (human calvaria, primary culture)	Bone	[87]
Chitosan/ nano-HA	3D porous blocks	Freeze-drying	Osteoblast-like MC3T3-E1 (newborn mouse calvaria cell line)	Bone	[278]
Chitosan/ β-TCP	3D porous blocks	Freeze-drying	Osteoblast-like MG63 (human osteosarcoma cell line)	Bone	[279,280]
Chitosan/ coralline	3D porous cylinders	Freeze-drying	MSCs: CRL-12424 (mouse bone marrow cell line)	Bone	[281]
Chitosan/ gelatin/HA	3D porous disks	Freeze-drying	Osteoblasts (neonatal rats calvaria, primary culture)	Bone	[282]
Chitosan/ gelatin/ (HA)	3D porous disks	Freeze-drying	MSCs (human bone marrow, primary culture)	Bone	[283]
Chitosan	3D porous disks	Freeze-drying	Chondrocytes (pig knee and dog shoulder joint, primary culture)	Cartilage	[77,284,285]
Chitosan	3D porous cylinders	Particle aggregation	ADAS cells (human adipose tissue, primary culture)	Cartilage	Malafaya, Pedro *et al.*, 2006
Chitosan/ polyester	3D fiber meshes	Fiber extrusion	Chondrocytes (bovine knee, primary culture)	Cartilage	[202,274,275]
Chitosan/ gelatin	3D porous cylinders	Freeze-drying	Chondrocytes (rabbit knee and pig auricular cartilage, primary culture)	Cartilage	[78,287]

(*Continued*)

Table 16.2 Chitosan-Based Scaffolds for Different Tissue Engineering Applications—Cont'd

Material	Scaffold Structure	Processing Methodology	Cell type (Source)	TE Application	References
Chitosan/ GP	Injectable gel	Gelation by polyol salts	Chondrocytes (calf knee, primary culture)	Cartilage	[288,289]
Chitosan/ hyaluronan	3D fiber sheets	Wet spinning	Chondrocytes (rabbit knee, hip, and shoulder joints, primary culture)	Cartilage	[79]
Chitosan/ alginate	3D porous cylinders	Freeze-drying	Chondrocyte-like HTB-94 (human bone chondrosarcoma cell line)	Cartilage	[43]
Chitosan/ (HA)	Bilayered	Particle aggregation	ADAS cells (human adipose tissue, primary culture)	Osteochondral	[72]
Chitosan/ hyaluronan	Bilayered with PLA	Freeze-drying	*In vivo* (rabbit femoral condyle)	Osteochondral	[154]
Chitosan	Porous membranes	Freeze-drying	-	Skin	[64]
Chitosan/ gelatin	Bilayered porous membranes	Freeze-drying	Co-culture of fibroblasts and keratinocytes (human skin, primary culture)	Skin	[60,61]
Chitosan/ collagen	Porous membranes	Freeze-drying	Co-culture of fibroblasts and keratinocytes (human foreskin, primary culture)	Skin	[290]
Chitosan	Tubular system	Wire-heating and freeze-drying	–	Neural	[291]
Chitosan	Porous hollow conduits	Thermal-induced phase separation	Neuro-2a cells (mouse neuroblastoma cell line)	Neural	[292–294]
Chitosan derivative	Tubular system	Crab tendon treatment	*In vivo* (rat sciatic nerve)	Neural	[295]
Chitosan/ hyaluronan	3D fiber sheets	Wet spinning	Fibroblasts (rabbit patellar tendon, primary culture)	Ligament	[159,160]
Chitosan/ collagen	3D porous blocks	Freeze-drying	Hepatocytes (rat liver, primary culture)	Liver	[94]
Chitosan/ gelatin	3D hydrogel cylinders	Solvent casting	Respiratory epithelial cells (human tissue, primary culture)	Tracheal	[296]

(), with or without; HA, hydroxyapatite; b-TCP, b-tricalcium phosphate; GP, glycerophosphate disodium salt; PLA, polylactic acid; MSCs, mesenchymal stem cells; ROS, rat osteosarcoma; ADAS cells, adipose derived adult stem cells

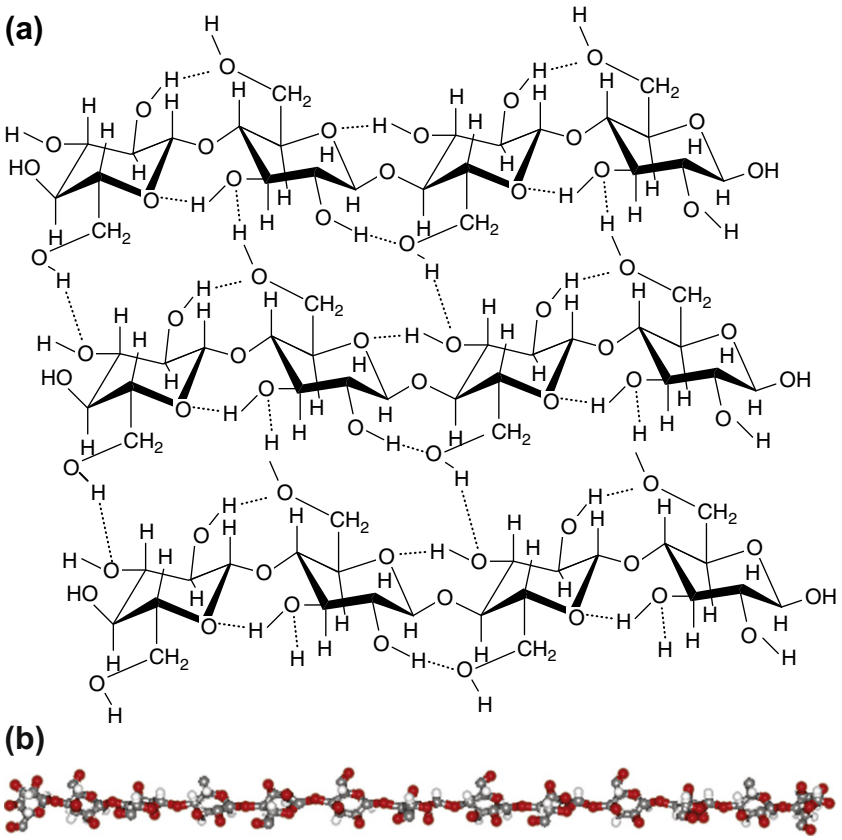


Figure 16.6 Structure of cellulose.

The biodegradability of cellulose is considered to be limited, if it occurs at all, because of the absence of hydrolases that attack the β(1→4) linkage [82]. This fact, together with the difficult processing, is the most limiting factor for the use of cellulose in tissue engineering applications. However, some partial degradation in processed cellulose sponges *in vivo* was reported [83]. The modification of the highly regular structural order of cellulose may also improve and tailor its degradation, as well as its tissue response [84]. For example, *in vitro* and *in vivo* studies on acetyl-cellulose and ethyl-cellulose sponges allowed to conclude that, for the first case, a gradual degradation over time could be detected, consistent with the observation on implanted sponges in Wistar rats [85].

Cellulose and its derivatives have been employed with success as biomaterials, and there are some indications that they could be an adequate source for tissue engineering applications. In orthopedic applications, it has been shown that cellulose sponges could support bone tissue ingrowths, suggesting that it could be used in bone tissue engineering [86]. Takata *et al.* [87] compared different membranes for guided bone regeneration and, among other materials, cellulose that also exhibited the ability to induce cell migration. Cellulose acetate and cellulose scaffolds also showed to be interesting for cardiac tissue regeneration, as they could promote cardiac cell growth, enhancing cell connectivity and electrical functionality [81].

Bacterial cellulose (BC) is a biotechnological method for producing pure nanofibrillar cellulose structures that have high mechanical strength, high water content, and high crystallinity [88]. BC is excreted extracellularly by *Acetobacter xylinum* bacteria, and the pellicle formed has been proposed to be used in tissue engineering-related applications. The biocompatibility of BC was confirmed *in vivo* where subcutaneous implantations in rats did not show substantial inflammatory response [89]. BC was shown to be able to support the proliferation of bovine-derived chondrocytes, and thus suggested the potential for use in tissue engineering of cartilage [90]. Moreover, the adequate mechanical properties of BC pellicles and the fact that smooth muscle cells adhere to and proliferate onto it suggested that BC could also be attractive for tissue engineering of blood vessels [88].

The properties of cellulose can be highly altered with chemical modification (e.g., through the

substitution of the hydroxyl groups) allowing expansion and tailoring of the physical features and the response to tissues of this material. A few cellulose derivatives have been specifically proposed for tissue engineering purposes. For example, 2,3-dialdehydecellulose porous membranes were prepared from methylcellulose combining water-induced phase separation and salt leaching techniques; this material is biodegradable and has been used as a drug carrier. Human neonatal skin fibroblast cells attached and spread on these membranes [91]. Hydroxypropyl methylcellulose grafted with silanol groups was developed as an injectable and self-setting hydrogel, which could be used to deliver and fix cells into a site through a nonevasive procedure [92]. Chondrocytes from two different origins were found to maintain their viability and to proliferate when cultured into the hydrogel [92], indicating that cellulose derivatives could also be used as a carrier of chondrocytes in cartilage tissue engineering. Cellulose sulfate was found to be biocompatible and nonimmunogenic and also showed to be adequate to encapsulate cells, to be used, for example, in protecting pancreatic xenogeneic cells from the immune system as a potentially curative treatment option for diabetes [93].

Cellulose and its derivatives may be seen as a potential source of natural-based materials in tissue engineering applications. There is, however, more work to be done in order to enhance the degradation rate and to find more processing routes to produce scaffolds with controlled architectures.

16.3.4 Starch

Starch is the dominant carbohydrate reserve material of higher plants, being found in the leaf chloroplasts and in the amyloplasts of storage organs such as seeds and tubers [94]. Although there is a broad range of possible origins of native starch, most of the starch utilized worldwide comes from a relatively small number of crops, the most important being corn, potato, wheat, and tapioca, with smaller amounts from rice, sorghum, sweet potato, arrowroot, sago, and mung beans [94].

As a natural polymer, it has received great attention as a possible alternative to synthetic polymers in several applications, mainly for being one of the cheapest biopolymers, being totally biodegradable into carbon dioxide and water [95], and abundantly available [95–97].

Native starch is composed of granules of variable sizes and shapes depending on the source of the starch. Chemically, starch is a polysaccharide consisting only of homoglucan units [95,96]. Starch is constituted by α-D-glucose units, which can be organized to form two distinct molecules, amylose and amylopectin [94,95,98–100]. The typical structure of amylose consists of a linear, very sparsely branched, polymer basically linked by 1→4 bonds [95,96,98,100,101]. On the contrary, amylopectin is highly branched on multiple points of the backbone, and contains not only 1→4 bonds, but also 1→6 branching points, that tend to appear each 25–30 glucose units [95,98,100,101]. The correspondent molecular weights are around 10^5–10^6 for amylose and 10^7–10^9 g/mol for amylopectin [95,99]. The distinct molecular weights and degrees of branching of both molecules are responsible for the quite different properties of starch isolated from sources with diverse amylose/amylopectin relative ratios [95,96,98] (Fig. 16.7). Besides its two basic macromolecular constituents, traces of lipids, proteins, and minerals (mainly phosphates) may also be found on native starch [98].

One of the most important properties of native starch is its semicrystallinity. Depending on the source and the moisture content, the degree of crystallinity in native starch ranges between 15 and 50% [102]. The crystallinity of starch is due to amylose and amylopectin, but mostly depends on amylopectin. Even though amylopectin has a branched structure, the branches form double helices between branches. The starch granule has been found to have alternating crystalline and amorphous concentric layers. The amorphous may be due to areas where the α-1,6 branch points form the chains, while the crystalline regions arise when the joined α-1,4 joined branches intertwine with each other and form double helices [99], resulting in the formation of parallel crystalline lamellae [102].

Besides its use as a filler material, native starch must be modified by destructuring of its granular structure to find other applications. The destructuring agent is usually water. The disruption of the granule organization obtained with the combination of water and heat is termed “gelatinization” and is characterized by the swelling of starch, forming a viscous past with destruction of most intermolecular hydrogen links [96,98].

To be able to make a thermoplastic starch (TPS) that can be processed by conventional processing

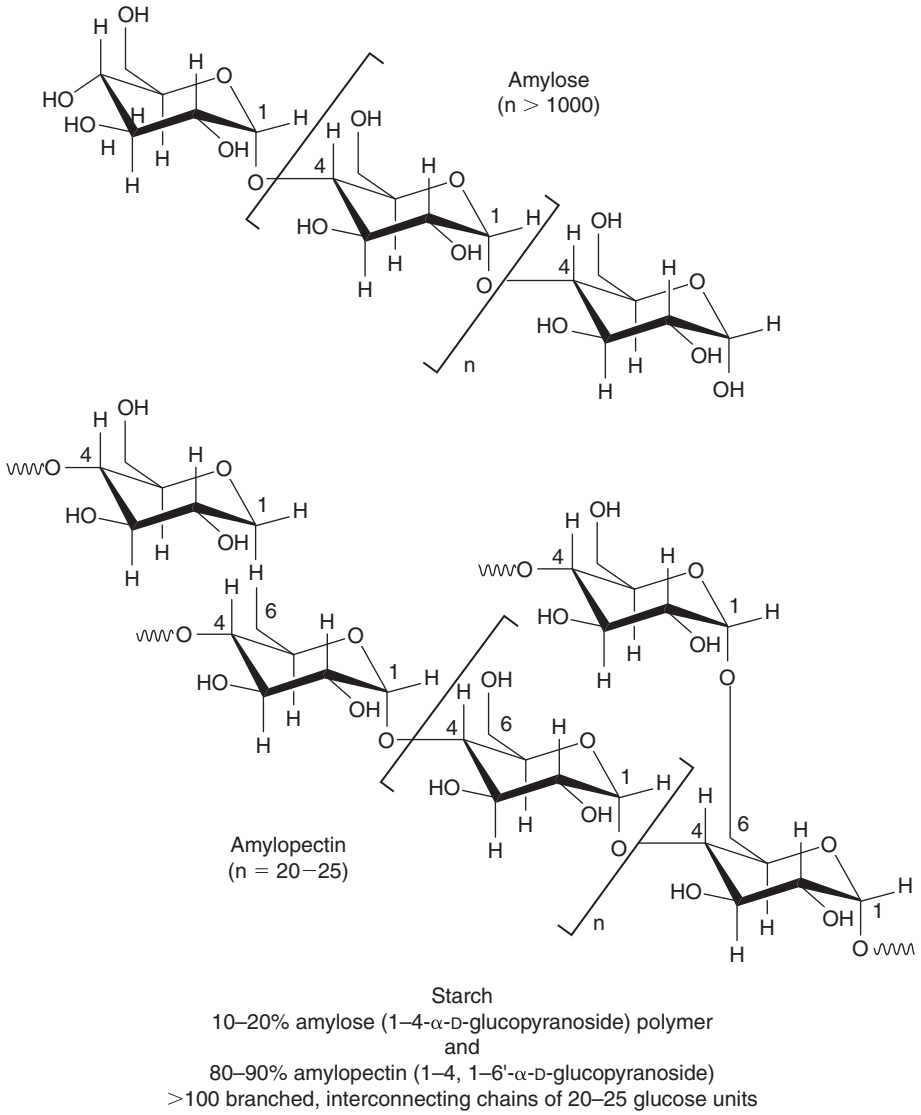


Figure 16.7 Structure of the two molecules that constitute starch, amylose, and amylopectin. The amylose content of starch can vary between 10 and 20% and the amylopectin content from 80 to 90%. The different ratios of amylose/amylopectin found in starch isolated from different sources, determine its properties.

techniques such as extrusion or injection molding, it is necessary to disrupt the granule and melt the partially crystalline nature of starch in the granule [98,101,103]. For granular starch, the glass transition temperature (T_g) is above the T_d of the polymer chains due to the strong interactions by hydrogen bonding of the chains. Several authors have estimated the T_g of dry starch to be in the region of 230−250 °C [98]. Therefore, plasticizers have to be added to lower the T_g beneath the T_d. Very important factors that will determine the final properties of TPS products are, among others, the type and amount of used plasticizers, the amylose/amylopectin ratio, the molecular weight of the starch (both mainly depend on the plant of origin), and the final crystallinity of the products [96,98,101]. Important plasticizers are water and several polyols such as glycerol and glycol [96,98].

Nevertheless, the application of unblended TPS is limited because of the thermal sensitivity and degradation of starch due to water loss at elevated temperatures [95,96]. Generally, for temperatures exceeding 180−190 °C, rapid degradation occurs during processing of TPS. The behavior of TPS is glassy and materials can only be processed by the addition of water, other plasticizers, or melt flow accelerators.

To overcome difficulties associated with the limited applicability of unblended TPS, while the starch is being destructurized in the extruder, it is possible to add, together with the plasticizers and other additives, other polymers in order to create biodegradable blends that will confer a more thermoplastic nature to the TPS. Other aimed properties are a better resistance to thermomechanical

Table 16.3 Examples of the use of Starch Based Polymers in Tissue Engineering Research

Polymer	Scaffold	Processing Methodology	Cells/Animal Model	TE Application	References
Starch/ polycaprolactone	2D porous scaffold	Selective laser sintering	NIH-3T3 mouse fibroblasts	Not specified	[43]
Starch/dextran/ gelatine	3D porous cylindrical	Rapid prototyping technologies (3D printing)	Not shown	Not specified	[297]
Starch/ polycaprolactone	Fiber mesh	Fiber bonding	Rat marrow stromal cells	Bone, cartilage	[134,271,298]
			Micro- and macrovascular endothelial cells	Bone	[299]
	Nano-micro fiber mesh	Fiber bonding 1 electrospinning	Rat marrow stromal cells	Bone	[271]
Starch/ethylene-vinyl alcohol	3D porous	Extrusion/injection molding with blowing agents	Human osteoblast-like cells (SaOS-2)	Bone	[109,111, 132,133,300]

degradation, meaning that the blends are more readably processable, have a less brittle nature, and enhanced resistance to water and ageing, as compared to fully starch thermoplastics. This has led to the development of a large range of starch-based thermoplastic blends for several different applications, including in the biomedical field.

Reis *et al.* [103,101,108,109,110,176,179,186, 200,201,233,234,235,281] have worked extensively using blends of corn starch (in amounts varying from 30 to 50%wt) with several different synthetic polymers such as poly(ethylene vinyl alcohol) (SEVA-C), cellulose acetate (SCA), poly(ϵ-caprolactone) (SPCL), and polylactic acid (SPLA) [104−106]. These polymers can be designed into distinct structural forms and/or properties by tailoring the synthetic component of the starch-based blend, their processing methods, and the incorporation of additives and reinforcement materials. These polymeric blends are degraded by hydrolytic processes and several enzymes [107,108] can also be involved in the process, mainly α-amylase, β-amylase, α-glucosidase, and other debranching enzymes [108]. The biocompatibility and nonimmunogenicity of starch-based polymers have been well demonstrated by several *in vitro* [109−111] and *in vivo* studies [110,112]. For all these reasons, starch-based polymers have been suggested for a wide range of biomedical applications, such as partially degradable bone cements [113−117], as systems for controlled release of drugs [109,117−119], as bone substitutes in the orthopedic field [120−124], and as scaffolds for tissue engineering [111,125−132]. A wide range of starch-based scaffolds have been developed exhibiting different properties and porous architectures, using several different processing methodologies, from conventional melt based technologies, such as extrusion and injection molding using blowing agents [130,133] to innovative techniques, such as microwave baking [130]. Some of these scaffolds have been successfully used in bone tissue engineering studies using human osteoblasts [111,132] and rat bone marrow stromal cells [128,134,135]. These and some other examples of tissue engineering studies performed using starch-based polymers as scaffold materials are summarized in Table 16.3.

16.3.5 Hyaluronan

Hyaluronan, formerly known as hyaluronic acid (HA), is a natural and highly hydrophilic polysaccharide, which has been found to be a key constituent of native ECM and tissues. HA belongs to the family of GAGs and is synthesized as a large,

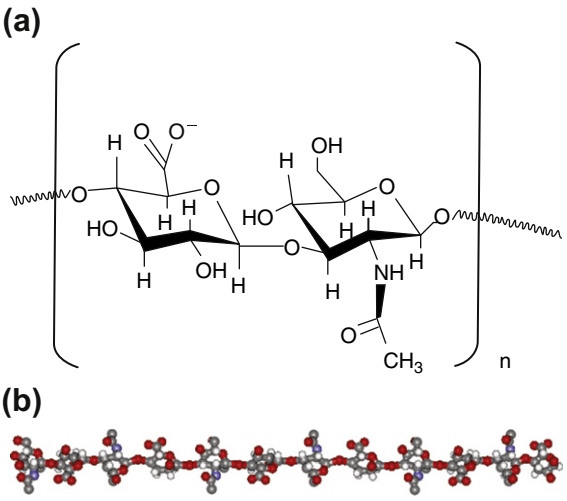


Figure 16.8 Structure of hyaluronan, which is composed of a repeating disaccharide of (1→3) and (1→4)-linked β-D-glucuronic acid and *N*-acetyl-β-D-glucosamine units.

negatively charged and linear polysaccharide of varying chain length (2225 μm) composed of repeating disaccharide units (Fig. 16.8) [136].

It is believed that interactions between HA with other ECM macromolecules and chondrocytes on the one hand, and its hydrodynamic characteristics, especially its viscosity and ability to retain water on the other, are critical for the maintenance of both cartilage homeostasis and biomechanical integrity. The characteristics of HA are, to a great extent, responsible for the regulation of the porosity and malleability of these matrices. Moreover, in the human body, HA is cleaved by enzymes called hyaluronidases [137], showing that the cells of the host or the ones present in the engineered tissue, may regulate the local clearance of the material, while the new tissue is being formed. Moreover, it has been demonstrated that HA is biocompatible [138] and has a greater bacteriostatic effect when compared with other matrices such as collagen type I, poly(lactide-*co*-glycolide) (PLGA), and hydroxyapatite [139].

Ehlers *et al.* [140,141] reported a double action of HA on chondrocytes, when added to the culture media. Results showed that chondrocytes presented a great tendency to differentiate and a higher rate of proliferation. These findings are corroborated by the works of other authors, which demonstrated that HA also stimulates bone marrow stromal cells proliferation and differentiation. These results are quite interesting since it is known that usually a differentiation-inducing stimuli leads to a lower cell proliferation. Therefore, HA possesses some of the features required when choosing a material suitable for tissue engineering scaffolding, although little is known about the mechanical properties of the HA molecules. The pioneering work of Fujii *et al.* [142] has demonstrated that the persistent length of single hyaluronan molecule is about 4.5 nm. This is important data for designing tissue engineering scaffolds, where the mechanical component is essential.

It was also found that HA interacts with cell surfaces in two ways, by binding to specific cell-surface receptors such as the hyaluronan receptor CD44 [143] and receptor for hyaluronan-mediated motility (RHAMM), and sustained transmembrane interactions with its synthetases [136]. The binding of chondrocytes to HA through the CD44 receptor greatly affects the functioning of these cells, thus cartilage homeostasis. In fact, the blocking of the CD44 receptors of chondrocytes results in the degradation of cartilage matrix. However, the physiological role of HA is not restricted to its participation in the synovial fluid of joints, umbilical cord, and vitreous body of the eye [137], but also has been described to be involved on the co-regulation of cell behavior during embryonic lung development, angiogenesis, wound healing processes, and inflammation [136]. In the last few years, HA and its derivatives have been showing interesting results when used in medicine, namely in the treatment of several soft and hard tissue defects such as skin [144,145], blood vessels [146,147], eye [148], ear [149], and bone [150] tissue. HA could certainly find other applications but the water solubility and rapid resorption preclude many clinical applications. To circumvent some of HA's limitations, several authors have been proposing the modification of the HA molecular structure, in an attempt to obtain more stable HA-based materials. Covalent cross-linking [151−153], partial or total esterification of its free carboxylic groups [149], and annealing [154] are allways to obtain a modified and stable form of HA. Most of hyaluronan-based polymers that can be obtained by cross-linking are water-insoluble gels or hydrogels (hylans), and much of which has still to be explored. In fact, HA and its derivatives offer a wide range of features that allow its prevalent use in tissue engineering as scaffolds since it can be used in the form of gels [155,156], sponges [157,158], films [151,153], fibers [159−161], and microparticles [162]. These materials met some of the

Table 16.4 Applications of Hyaluronan and Its Derivatives in Tissue Engineering

Polymer	TE Application	References
Hyaluronan/fibrin glue	Articular cartilage	[155]
Hyaluronan/alginate	Articular cartilage	[30,107,301]
Hyaluronan/chitosan	Articular cartilage and skin	[79,159,160,166]
Hyaluronan/collagen cross-linked via a poly(ethyleneglycol) diepoxide	Not specified	[164]
Hyaluronan/collagen cross-linked via pyridinoline	Articular cartilage	[302]
Hyaluronan modified with methacylic anhydride	Articular cartilage	[303]
Hyaluronan/elastin	Articular cartilage	[153,304]
Fibronectin-coated ACP™ (hyaluronan-based sponge)	Osteochondral	[158]
Hyaluronan/calcium phosphates	Bone and osteochondral	[150]
Hyaluronan/PLGA	Osteochondral and articular cartilage	[158,167]
Hyaluronan/laminin	Brain	[165]
Hyaff® (hyaluronan derivative obtained by esterifying the free carboxylic group)	Skin, cartilage, trachea, and other soft tissues	[149,157,161,305–309]
Laserskin® (hyaluronan 100% esterified with benzyl alcohol)	Skin	[144,145]
Disulfide-cross-linked hyaluronan	Soft tissues	[151]
Hyaluronan-graft-poloxamer	Eye	[148]
Hylans (hydrogels based on cross-linked hyaluronan)	Vascular and aortic heart valves	[146,147,310]
Nonwoven Hyaff (esterified Hyaluronan)	Vascular	[305,311]

criteria for their successful application not only in tissue engineering scaffolding [157], but also in drug delivery applications [148,162]. Despite this, a great deal of attention has been given to the development of alternative high-quality scaffolds. In this context, several authors have been proposing the blending with other polymers to utilize the benefits of each biomaterial. Hyaluronan has been combined with fibrin glue (useful cell delivery matrix) [155], alginate [163], collagen [164], gelatin [153], laminin [165], chitosan [79,166], polyesters [167], and calcium phosphates [150] to develop composite scaffolds for regeneration of several damaged tissues (Table 16.4).

It has been known for some time that HA also plays a key role in interactions with tumor cells [168]. In fact, there is an association of high levels of HA with malignancy of tumors [169]. These observations highlight the biological role of HA, demonstrating that this molecule can be a viable therapeutic target, which might be useful for developing more effective therapeutic strategies in the coming years.

16.4 Proteins

Proteins are the most abundant organic molecules within the cell extracellular and intracellular medium, where they ensure multiple biological functions, such as transport, regulation of pathways, protection against foreign molecules, structural support, protein storage, as well as being the catalyst for a great diversity of reactions, acting as biocatalysts (enzymes).

In a molecular perspective, proteins may be considered as polymer structures composed of 20 distinct amino acids linked by amide (or peptide) bonds. Amino acids are, therefore, the building blocks of polypeptides and proteins, which consist of a central carbon linked to an amine group, a carboxyl group, a hydrogen atom, and a side chain (R groups). R groups can be classified as nonpolar groups, uncharged polar groups or charged polar groups, in which their distribution along the protein backbone renders proteins with distinct characteristics.

The structure of a protein is not, however, as simple as a polysaccharide, or other polymer. Generally, the protein structure is described on four levels. The *primary structure* of a protein is its amino acid sequence, whereas the *secondary structure* refers to the local spatial arrangement of the polypeptide's backbone atoms without regard to the conformation of its side chains. The folding of the polypeptide chain is responsible for putting in close contact different parts of the chain to create binding sites to the substrate, etc. The *tertiary structure* is related to the 3D structure of the entire polypeptide. When proteins are composed of more than one polypeptide chain (referred as subunits), the resultant spatial arrangement of its subunits is known as the protein's *quaternary structure*.

The configuration assumed by a protein, and thus the one that determines its properties, is the one that minimizes the molecule's free energy. Protein conformation is determinant for protein bioactivity, being known that a certain 3D structure is essential for protein functionality. Most of the forces that stabilize the protein structure are weak (hydrogen bonding, ionic and hydrophobic interactions, van der Waals forces), giving some flexibility to the macromolecule. In general, nonpolar amino acid side chains (e.g., phenylalanine, leucine, tryptophan, valine, etc.) are located in the interior of the protein away from the aqueous solvent. The hydrophobic effects that promote this distribution are largely responsible for the 3D structure of native proteins. On the contrary, ionized side chains tend to be on the surface of the molecule to interact with the aqueous solvent. In addition, the polypeptide chains of larger proteins tend to exist in structural domains independently folded and connected by segments of peptide chains.

Taking into account the low stabilities of protein conformations, these molecules are easily susceptible to denaturation by changing the balance of the weak interactions that maintain the native conformation. Proteins can be denaturated by a variety of conditions and substances as heating, extreme pH, chaotropic agents, detergents, adsorption to certain surfaces, etc. [170].

We now focus on the most important proteins that have been studied for tissue engineering applications, such as collagen, elastin, soybean, and silk fibroin.

16.4.1 Collagen

Collagen is the most abundant protein in mammalian tissues (cornea, blood vessels, skin, cartilage, bone, tendon, and ligament) and is the main component of the ECM [171,172]. Its main function is to maintain the structural integrity of vertebrates and many other organisms. However, collagens also exert important functions in the cell microenvironment and are involved in the storage and release of cell mediators, like growth factors [171]. More than 20 genetically distinct collagens have been identified [171−174], but the basic structure of all collagens is composed of three polypeptide chains, which wrap around one another to form three-stranded rope structure (triple helix, Fig. 16.9b). Close-packing of the chains near the central axis imposes the requirement that glycine (Gly) occupies every third position, generating a (X-Y-Gly)*n* repeating sequence. Proline (Prol) and 4-hydroxyproline (Hyp), which in collagens constitute about 20% of all residues, are found almost exclusively in the X and Y positions, respectively. Therefore, the most common triplet in collagen is Prol-Hyp-Gly, which accounts for about 10% of the total sequence [175] (Fig. 16.9a). Peptides that contain Gly as every third residue and have large amounts of Prol and Hyp behave as triple helices in solution.

The individual triple helices are arranged to form fibrils which are of high tensile strength and can be further assembled and cross-linked (collagen fibrils are stabilized in the ECM by the enzyme lysyl oxidase).

In tissues that have to resist shear, tensile, or pressure forces, such as tendons, bone, cartilage, and skin, collagen is arranged in fibrils, with a characteristic 67 nm axial periodicity, which provides the tensile strength [176]. Only collagen types I, II, III, V, and XI self-assemble into fibrils [171]. The fibrils are composed of collagen molecules, which consist of a triple helix of approximately 300 nm in length and 1.5 nm in diameter [176]. Collagen fibril formation is an extracellular process, which occurs through the cleavage of terminal procollagen peptides by specific

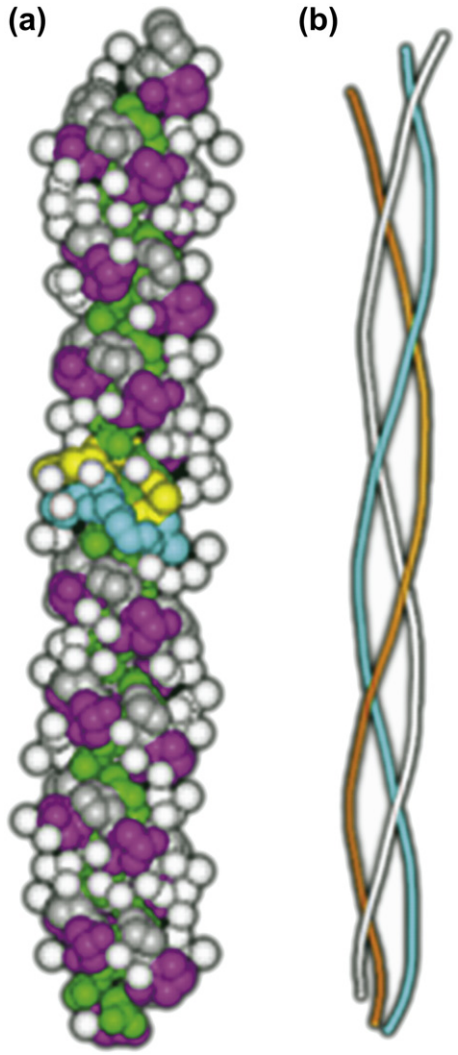


Figure 16.9 (a) CPK model of the structure of the triple-helical collagen-like peptide (1QSU, retrieved from Protein Data Bank at http://www.rcsb.org) showing Gly residues in green, Prol resides in gray and Hyp (magenta). All residues are exposed to the solvent (water molecules are displayed in white); (b) Schematic representation of the collagen-like peptide showing the triple helix. The structures were generated using the WebLab ViewerLite 3.7 program (Molecular Simulations Inc, USA).

procollagen metalloproteinases. An article by Stevens and George [177] provides an interesting schematic diagram showing the natural assembly of collagen fibers. Further reading about collagen fibril formation and molecular packing in collagen fibrils may be found in previous publications [178−180].

During the 1970s and 1980s, academics and commercial researchers began to use collagen as a biomaterial in a variety of connective tissue applications because of its excellent biocompatibility [173,174], low antigenicity [173,174], high biodegradability [173,181,182], and good hemostatic and cell-binding properties [173,183].

The primary sources of industrial collagens are from animal tissues (porcine and calf skin, bovine tendon, rat tail, etc.). It may readily be purified from animal tissues with enzyme treatment and salt/acid extraction. However, the use of animal-derived collagen raises concerns over the possible transmission of infectious agents such as viruses and prions [172]. Transmissible bovine spongiform encephalopathy (BSE) is one of the most difficult contaminating agents to detect and remove from animal tissues. Therefore, different attempts have been made to find new and safer sources of collagen, namely from marine sources (e.g., jellyfish collagen) [184] or by producing recombinant human collagen (rhC) for clinical use [172] using different expression systems. The use of recombinant sources of human collagen provides a reliable, predictable, and chemically defined source of purified human collagens that is free of animal components (please see for instances the review of Yang *et al.* [172]; for more details about the application of rhC in tissue engineering). The triple-helical collagens made by recombinant technology have the same amino acid sequence as human tissue-derived collagen. In addition, collagen products can be purified from fibers, from molecules reconstituted as fibers, or from specific recombinant polypeptides with specific composition and conformation.

A feature common to all of these collagen materials is the need for stable chemical cross-linking to control the mechanical properties and the residence time in the body, and to some extent their potential immunogenicity. This can be achieved via chemical (glutaraldehyde, formaldehyde, carbodiimides, diphenylphosphoryl azide), physical (UV radiation, freeze-drying, heating, thermal dehydration), and enzymatic cross-linking. These cross-linking agents react with specific amino acid residues on the collagen molecule imparting individual biochemical, thermal, and mechanical stability to the biomaterial. Collagen may be an ideal scaffold material, as it is the major component of the ECM, and because it can be processed into a wide variety of structures and shapes (sponges, fibers, films, 3D gels, fleeces; Table 16.5). Furthermore, collagen substrates can modify the morphology, migration, and in certain cases the

Table 16.5 Examples of Application of Collagen Scaffolds in Tissue Engineering Research

Type of Scaffold	Processing Methodology	TE Application	References
Collagen sponge with 11 mm in diameter and 2 mm in thickness and pore volume fraction of 97.5%	Freeze-drying. Cross-linking by thermal dehydration	Tooth tissue engineering Guided tissue regeneration (GTR) in dentistry	[312]
Collagen membranes	Conversion of rhCI monomers into oligomers and reconstitution into collagen fibrils. The resulting fibrillar networks were subsequently cross-linked with ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)		[172]
Scaffolds with predefined and reproducible internal channels with widths of 135 μm	Rapid prototyping (solid freeform fabrication technology and critical point drying technique)	Cardiovascular (aortic valve, blood vessel) tissue engineering	[313,314]
Scaffolds of 6 mm in diameter and 0.75 mm in thickness	Freeze-drying, cross-linked with hexamethylene diisocyanate		[182]
Flat sheets of collagen type I	Solvent evaporation		[183]
Porous tubular scaffolds with an inner diameter of 3 mm, an outer diameter of 6 mm and a length of 4 cm	Freeze-drying of a suspension of type I insoluble collagen and insoluble elastin. Cross-linked with a carbodiimide		[315,316]
Collagen-gel tubular constructs	Polymerization into glass test tubes		[317]
Type I collagen sponge with interconnected pores	Discs cored from sheets of Ultrafoam® collagen hemostat (Davol Inc., Cranston RI)	Bone tissue engineering Bone graft substitutes	[318]
Recombinant collagen sponges (porous micromatrice structures interconnected by homogenous thin sheets of recombinant human collagen I fibrils)	In-mold fibrillogenesis/cross-linking process followed by lyophilization. Cross-linking with EDC		[172]
Collagen gel (Atelocollagen gel from Koken Co., Tokyo, Japan). Dome shape of 0.8 cm diameter and 0.2 cm top height	Gelation at 37 °C for 60 min	Cartilage tissue engineering	[319]
Matriderm®. 3D structure made of purified collagen I of bovine epidermis and small amounts of elastine	Freeze drying. Cross-linking with a carbodiimide		[320]
Collagen-based wound dressings (membranes, fibers, sponges)	Several	Dermal tissue engineering (artificial skin, skin substitutes)	[180]

(*Continued*)

Table 16.5 Examples of Application of Collagen Scaffolds in Tissue Engineering Research—Cont'd

Type of Scaffold	Processing Methodology	TE Application	References
Type I collagen contracted gels (discs)	Gelation at 37 °C for 60 min		[321]
Fibrous scaffolds	Electrospinning. Cross-linking with 1,6-diisocyanatohexane	TE applications in general	[187]

differentiation of cells due to the presence of cell adhesion sequences present on its structure (e.g., RGD). Collagen is naturally degraded by matrix metalloproteinases, specifically collagenase, and serine proteases [180]. These enzymes are secreted by neutrophils during the foreign body reaction, allowing the collagen degradation to be controlled by the cells present at the implantation site [182]. However, its low thermal stability, due to its protein nature, does not allow collagen to be processed by melt-based techniques, limiting its processing to solvent-based methods and consequently the final properties of the scaffolds, normally characterized by poor mechanical strength. Another drawback related with the use of collagen materials is the requirement of additional chemical or physical cross-linking to confer mechanical strength and enzymatic resistance. Intermolecular cross-linking reduces the degradation rate by making collagen less susceptible to enzymatic attack. Collagen has long been known to elicit minimal inflammatory and antigenic responses and has been approved by the United States Food and Drug Administration (FDA) for many types of medical applications, including wound dressings and artificial skin [180,185]. These properties of collagen emphasize its significance in tissue regeneration and its value as a scaffold material, being currently used in a great number of tissue engineering applications. Table 16.5 presents some examples of TE applications using collagen scaffolds. In addition, composites of collagens with GAGs, as well as with synthetic biodegradable polymers and ceramics, have also been extensively studied for their potential application as scaffolds for tissue engineering. A vast number of publications can be found in the literature, covering a diversity of clinical applications, such as general surgery, orthopedics, cardiovascular, dermatology, otorhinolaryngology, urology, dentistry, ophthalmology, and plastic and reconstructive surgery [180].

Although these examples offer encourage applications of collagen in tissue engineering, its low mechanical properties, the risk of viral infection, its antigenicity potential, and fast biodegradation when implanted in the human body are, to some extent, limiting the clinical applications of this natural biomaterial.

16.4.2 Elastin

Elastin is another key structural protein found in the ECMs of connective tissues (e.g., blood vessels, esophagus, skin) that need to stretch and retract following mechanical loading and release [186,187]. It is found predominantly in the walls of arteries, lungs, intestines, and skin, as well as other elastic tissues. However, unlike type I collagen, elastin has found little use as a biomaterial, for two main reasons [188,189]: (i) elastin preparations have a strong tendency to calcify upon implantation, probably because of the microfibrillar components (mainly fibrillin) within the elastic fiber that are difficult to remove and (ii) the purification of elastin is complex [188]. The insoluble nature of elastin has also limited in its use in traditional reconstituted matrix fabrication techniques [190] and when applied only poorly defined elastin preparations have been used [189].

Elastin consists of several repetitive amino acid sequences, including VPGVG, APGVGV, VPGF GVGAG, and VPGG [191]. Highly insoluble and extensively cross-linked, mature elastin is formed from tropoelastin, its soluble precursor [192]. Tropoelastin is secreted from elastogenic cells as a 60-kDA monomer that is subjected to oxidation by lysyl oxidase. Subsequent protein−protein associations give rise to massive macroarrays of elastin [187]. The structure of tropoelastin consists of an alteration of hydrophobic regions, responsible for elasticity, and cross-linking domains. Additionally, it ends with a hydrophilic carboxy-terminal sequence containing its only two cysteine

residues [192]. As a consequence, elastin is a substantially insoluble protein network [187,192]. Soluble material is typically derived either as a fragmented elastin in the form of alpha- and kappa-elastin or preferably through expression of the natural monomer tropoelastin [187]. In the production of α-elastin, bovine ligament elastin is treated [193] with a mild acid hydrolysis to yield a high-molecular-weight digest that retains the amino acid composition of native elastin. Despite structural heterogeneities resulting from the hydrolysis, α-elastin retains several key physicochemical properties of the nascent elastin. Nevertheless, the development of α-elastin based biomaterials is still a quite unexplored area (Fig. 16.10).

Recombinant protein technologies have allowed the synthesis of well-defined elastin-derived polypeptides, which have driven insightful structure-function studies of tropoelastin, as well as several discrete elastin domains. Elastin-like polypeptides (ELPs) are artificial polypeptides with unique properties that make them attractive as biomaterial for tissue engineering, as it has been demonstrated by the work of Urry *et al.* [63,194,195]. ELPs consist of oligomeric repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly (Xaa is any amino acid except proline), a naturally occurring sequence in the protein elastin. ELPs are soluble in aqueous solution below their transition temperature (T_t) but when the solution temperature is raised above their T_t, the polymers start a complex self-assembly process that leads to an aggregation of the polymer chains, initially forming nano- and microparticles, which segregate from the solution [77,196]. This "smart" nature may not be of particular interest for the final application of ELPs as ECM, but it is extremely important to simplify several steps in the production of ELPs and preparation of the ECM [197]. ELPs have also demonstrated an outstanding biocompatible behavior. Apparently, the immune response system of the human body does not differentiate the ELPs from endogenous elastin. Moreover, because of their protein nature, their bioabsorption is carried out by conventional metabolic routes, yielding just natural amino acids [197]. In addition, the matrices resulting from cross-linking of ELPs show a mechanical response quite similar to the natural elastin [198]. This characteristic is very important for their application in tissue engineering, as the scaffold (artificial ECM) has to properly transmit the forces from the surrounding environment to the attached cells so that they can build new tissue that can eventually replace the artificial ECM [197].

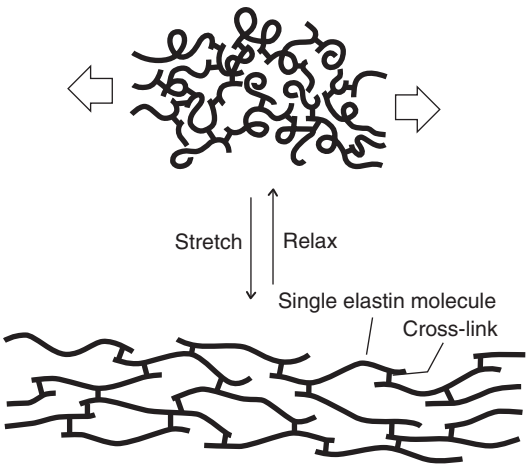


Figure 16.10 Structure of elastin.

However, the broad application of these materials is limited by the inherent challenges of synthesizing recombinant proteins (e.g., residual endotoxin, capital cost and expertise, scale-up).

Table 16.6 gives several examples of tissue engineering studies in which elastin-based scaffolds were used.

16.4.3 Soybean

Soybeans belong to the legume family and can be processed into three kinds of protein-rich products: soy flour [199,200], soy concentrate [200,201], and soy isolate [200,203]. Soy protein, the major component of the soybean (30–45%) is readily available from renewable resources, is economically competitive, and presents good water resistance as well as storage stability [204]. About 90–95% of the soy is storage protein, with two subunits, namely 35% conglycinin (7S) and 52% glycinin (11S) [205]. Due to its low cost and surface active properties, soy protein is of great importance to the food industry, especially as it provides stability against phase separation in food systems [206]. Nevertheless, the combination of its properties with a similarity to tissue constituents and a reduced susceptibility to thermal degradation makes soy an ideal template for use in biodegradable polymer for biomedical applications [207]. Membranes, microparticles, and thermoplastics-based soy materials have been developed for tissue regeneration [208]. Biodegradable soy plastics have been developed by melt-based methods

Table 16.6 Examples of Application of Elastin Based Scaffolds in Tissue Engineering Research

Polymer	Scaffold	Processing Methodology	Cells/Animal Model	TE Application	References
α-Elastin	Films	Cross-linking	Bovine aortic smooth muscle cells	Vascular tissue	[193]
Elastin and tropoelastin	Fibers	Electrospinning followed by cross-linking	HEPM	Not specified	[187]
Aortic elastin	3D porous structure	Cyanogen bromide treatment for decellularization and removal of collagen and other ECM components	3T3 mouse fibroblast cell line (ATCC)	Not specified	[322]
Collagen/ elastin/PLGA	Electrospun fiber meshes	Electrospinning	Bovine endothelial and smooth cells	Vascular tissue	[323]
Elastin/ collagen	3D structure composed of thin sheets and fibrils (collagen) and thick fibers (elastin)	Lyophilization followed by cross-linking	Sprague-Dawley rats (subcutaneous pockets	Not specified	[188]
Aortic elastin	3D porous structure	Cyanogen bromide treatment (CNBr)	Sprague-Dawley rats (subdermal implantation)	Vascular tissue	[73]
Collagen and elastin (1:1)	Tubular porous structures	Freeze-drying followed by cross-linking	Human smooth muscle cells	Vascular tissue	[315,316,324]
Elastin-like polypeptides (ELPs)	Injectable scaffolds	Gene design and synthesis	Pig chondrocytes	Cartilage	[77,196]

PLGA, poly(D,L-lactide-co-glycolide); HEPM, human embryonic palatal mesenchyme

such as extrusion and injection molding [207]. Mano *et al.* [207] reported that soy protein-based thermoplastics presented a suitable range of mechanical and dynamical properties that might allow their use as biomaterials, namely in controlled release applications.

Soy protein has many reactive groups, such as $2NH_2$, 2OH, and 2SH, that are susceptible to chemical and physical modifications [209]. Some studies reported that the combination of soy protein with other proteins (e.g., wheat gluten [210], casein [207], and polysaccharides) such as cellulose [97], dialdehyde starch [211], and chitosan [212,213] in film form may promote physical and chemical interactions, which improve some properties. Silva *et al.* [214] have reported that, by means of combining a sol−gel process with the freeze-drying technique, it was possible to develop cross-linked porous structures based on chitosan and soy protein. It was demonstrated that the developed porous structures possess a suitable porosity and adequate interconnectivity. Furthermore, tetraethylorthosilicate (TEOS) can be used to introduce specific interactions in the interfaces between chitosan and soy protein, and improve its mechanical stability and degradability. Therefore, this work has shown that these structures have great potential for tissue engineering of cartilage.

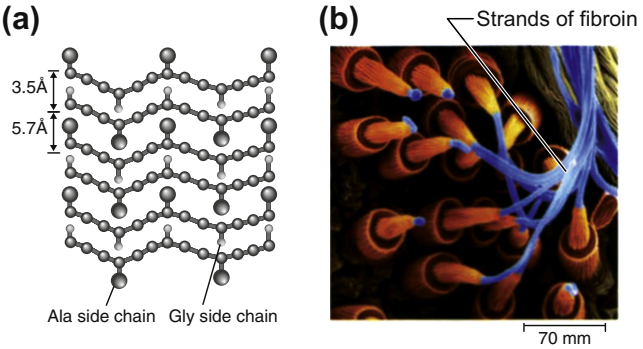


Figure 16.11 Structure of silk. The fibers used to make silk cloth or spiderweb are made up of the protein fibroin. (a) Fibroin consists of layers of antiparallel β sheets rich in Ala (purple) and Gly (yellow) residues. The small side chains interdigitate and allow close packing of each layered sheet, as shown in this side view. (b) Strands of fibroin (blue) emerge from the spinnerets of a spider in this colorized electron micrograph. *Source: Nelson, D.L. and Cox, M.M. (2003). Lehninger Principles of Biochemistry, 3rd edn, Worth Publishers, New York, NY, p. 174.*

16.4.4 Silk Fibroin

Silk fibroin is a highly insoluble fibrous protein produced by domestic silk worms (*Bombyx mori*) containing up to 90% of the amino acids glycine, alanine, and serine leading to antiparallel β-pleated sheet formation in the fibers [215]. Fibroin is a structural protein of silk fibers and sericins are the water-soluble glue-like proteins that bind the fibroin fibers together [216] (Fig. 16.11). High purity silk fibroin fiber can be obtained easily from degummed silk (boiling-off), which refers to partial or complete removal of the sericin. Removal of the sericin coating before use removes the thrombogenic and inflammatory response of silk fibroin [217]. *Bombyx mori* silk fibroin can be dissolved with neutral salt solutions such as lithium bromide (LiBr), lithium thiocyanate (LiSCN), hexafluoroisopropyl alcohol (HFIP), and calcium nitrate-methanol [$Ca(NO_3)_2$-MeOH] [218]. Their mixtures are dialyzed to get pure fibroin solution, which can be used to prepare silk fibroin membranes, fiber, hydrogel, scaffolds, and others types of materials [216]. Traditionally, silk fibroin has been used for decades as suture material [216]. Nowadays, several studies demonstrate the utility of silk matrices in films [216,217,219], nanofibers [215,220,221], hydrogels [222], and porous matrices [223,224] for biomaterials and tissue engineering with stem cells for cartilage and bone applications. These applications of silk fibroin are related to its permeability to oxygen and water, cell adhesion and growth characteristics, slow degradability, low inflammatory response, and high-tensile strength with flexibility [216]. Porous 3D scaffolds with silk fibroin have been obtained using various processing techniques (Table 16.7); these include salt leaching [223–225], electrospinning [220,221,226], freeze-drying [224,227–230], and gas-foaming [224]. Li *et al.* [228–230] reported a series of studies on preparation conditions of porous silk fibroin materials and its relationship between the structure and properties. These materials were prepared by means of freeze-drying. A new process to form a silk fibroin spongy porous 3D structure with both good porous structures and mechanical properties has also been reported [231]. This process involves freezing and a thawing fibroin aqueous solution in the presence of a small amount of water-miscible organic solvent. It requires no freeze-drying, no cross-linking chemicals, or the aid of other materials. In general, the silk scaffolds produced by different methods

Table 16.7 Examples of Application of Soy and Silk-based Materials in Tissue Engineering Studies

Polymer	Scaffold	Processing Methodology	TE Application	References
Soy protein/ chitosan blend	Not specified	Freeze-drying and sol–gel process	Cartilage	[214]
Silk fibroin	Not specified	Freeze-drying	Not specified	[227–230]
Silk fibroin	Not specified	Salt leaching	Cartilage, bone	[225,235,236]
Silk fibroin	Not specified	Gas-foaming	Not specified	[224]
Silk fibroin	Nanofiber	Electrospinning	Bone	[226]

Source: Invited chapter, in: Tissue Engineering, Clemens van Blitterswijk, Anders Lindahl, Peter Thomsen, David Williams, Jeffrey Hubbell, Ranieri Cancedda (Editors), Elsevier.

described here presented good porosity and mechanical properties that can be controlled by silk fibroin concentration, freezing temperature, and particle size of salt used in the process. Other approaches to form silk scaffolds involved the blending of polymers, such as poly(ethylene oxide) [215], chitosan [227], or the surface modification of synthetic polymers such as poly(ε-caprolactone) [232] and polyurethane [233] with silk fibroin coating in order to improve their collective properties, especially processability, mechanical properties, and biocompatibility, respectively.

With respect to using silk fibroin for cell culture, many researchers have investigated the effects of the silk matrices in nanofiber and porous matrix obtained by methodologies described previously on the culture of osteoblasts-like cells [234], human mesenchymal stem cells [221,225,226,235,236] have shown very promising results regarding their application in cartilage and bone tissue engineering. Jin *et al.* [221] concluded that electrospun silk matrices support bone marrow mesenchymal stem cells attachment, spreading, and growth *in vitro*. Meinel *et al.* [235] reported the feasibility of silk-based implants with engineered bone for the (re-)generation of bone tissues. Recently, the potential of electrospun silk fibrous scaffold for bone formation from human bone marrow-derived mesenchymal stem cells (hMSCs) was explored by combining the unique structural features generated by electrospinning with functional factors, such as bone morphogenic protein-2 (BMP-2) and nanohydroxyapatite particles [226].

16.5 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are naturally occurring biodegradable polymers. PHAs are synthesized and stored as water-insoluble inclusions in the cytoplasm of several bacteria and used as carbon and energy reserve materials [237,238]. The first PHA to be identified was poly(3-hydroxybutyric acid) (P[HB]). This homopolymer is the most abundant bacteria synthesized polyester and its 3-hydroxybutyrate (HB) monomer was thought to be the unique PHA constituent in bacteria [238,239]. Further research [240] reported heteropolymers in chloroform extracts of activated sewage sludge, like 3-hydroxyvalerate (HV) among others. The introduction of other units in the PHA chain (besides 3HB) has a significant effect on the mechanical

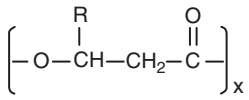


Figure 16.12 Structure of polyhydroxyalkanoates.

behavior of polyester [241,242]. The homopolymer of PHB is a brittle material, while the increase in HV content turns the HB-*co*-HV copolymer more ductile [243–245]. The mechanical behavior of PHAs depends on both the length of the pendant groups and the distance between ester linkages. PHAs with short pendant groups are prone to crystallization but exhibit stiff and brittle behavior, while PHAs with longer pendant groups are ductile [246]. The wide performance range of PHA copolymers justified additional scientific and industrial interest, which led to the discovery of further bacterial PHAs. PHAs can be synthesized in molecular weights that depend on the growth conditions and on the microorganism species—between, 200,000 and 3,000,000 Da [238] (Fig. 16.12).

The wide range of mechanical properties [243,245,247–254] coupled with the biodegradable [248,253–257] and the biocompatible behaviors [256,258–261] of PHAs makes them potential biomedical candidates including drug delivery and tissue engineering applications [246,257]. The biocompatibility assessment of PHAs has indicated that cell response also depends on the type of polyester. In a research study, the viable cell number of mouse fibroblasts (cell line L929) on polyhydroxybutyrate (PHB) films have increased more than two orders of magnitude upon blending with poly(hydroxybutyrate-*co*-hydroxyhexanoate) (PHBHH) [262]. The influence of PHB content on mechanical behavior is also evident from the strong ductility increase which occurs with the introduction of PHBHH in PHBHH/PHB blends [263]. Several studies [264–270] reported the investigation of PHAs as potential scaffold materials in diverse range of tissue engineering applications. Sodian *et al.* developed a trileaflet heart valve from a porous PHA scaffold produced by salt leaching. Constructs were produced using vascular cells harvested from an ovine carotid artery and placed into a pulsatile flow bioreactor [268]. Results indicated that cells were mostly viable and grew into scaffolds pores. The formation of connective tissue between the inside and the outside of the porous heart valve scaffold was also observed. Other studies have focused the assessment of PHA

scaffolds for bone and cartilage tissue engineering. A study by Rivard *et al.* [265] investigated the proliferation of ovine chondrocytes and osteoblasts in poly(β-hydroxybutyrate-β-hydroxyvalerate) scaffolds. Another study assessed the performance of porous PHBHH/PHB scaffolds, produced by salt leaching method, as matrices for 3D growth of chondrocytes. Cell densities were higher for PHBHH/PHB scaffolds as compared to PHB scaffolds alone. The authors explained this discrepancy based on eventual differences in crystalline and amorphous arrangements between PHB and PHBHH/PHB scaffolds, as the presence of PHB crystalline domains may reduce oxygen permeability [264]. Another study [270] has shown that PHB/PHBHH blends with 1:1 ratio have higher surface free energy as compared to PHB alone, which maximizes chondrocytes adhesion. Furthermore, polarity of the PHA on the scaffold seems to play an important role on what concerns cell morphology. In the case of PHBHH/PHB substrates, PHB content affects blend polarity, which has an important effect on cell shape. Polarity increases with decreasing blend crystallinity, which affects chondrocyte shape, by altering it from spherical to flat.

16.6 Future Developments

Several scaffolds based on natural origin polymers have been widely studied for tissue engineering application. Many of them exhibit unique advantageous features concerning intrinsic cellular interaction and degradability. However, these materials do also exhibit some disadvantages that limit their widespread use. Therefore, it is necessary to increase the knowledge about these natural polymers in order to enable the development of new approaches, including methods for production, purification, controlling material properties (molecular weight, mechanical, degradation rate), and for enhancing material biocompatibility (for instance by using nonanimal derived production), in order to design better and more versatile scaffolding materials.

Tissue engineering scaffolding will also benefit from advances in recombinant protein technologies, which have proven to be a very powerful tool for the design and production of complex protein polymers with well-defined molecular weights, monomer compositions, sequences, and stereochemistry. Very little has been explored within this new class of polymers and therefore much remains to be investigated about their versatility and possibilities of obtaining tailored properties for target applications. Accordingly, special interest has emerged for the use of these protein-based polymers for tissue engineering and other biomedical applications.

Further studies are expected to widen the range of natural origin materials (and combination of these with synthetic polymers) and the tailoring of their properties in order to make them even more suitable for applications within tissue engineering.

16.7 Summary

1. A wide range of natural origin polymers have frequently been used and might in future be potentially useful in tissue engineering.
2. Tissue engineering scaffolds comprised of naturally derived macromolecules have potential advantages of biocompatibility, cell-controlled degradability, and intrinsic cellular interaction.
3. However, they may exhibit batch variations and, in many cases, exhibit a narrow and limited range of mechanical properties. In many cases, they can also be difficult to process by conventional methods.
4. In contrast, synthetic polymers can be prepared with precisely controlled structures and functions. However, many synthetic polymers do not degrade as desired in physiological conditions, and the use of toxic chemicals in their synthesis or processing may require extensive purification steps. Many of them are also not suitable for cell adhesion and proliferation.
5. The combination of natural origin polymer with synthetic polymers and the further development in emerging methodologies such as recombinant protein technologies is expected to lead to outstanding developments in improved materials to be used in tissue engineering applications.
6. No one material alone will satisfy all design parameters in all applications within the tissue engineering field, but a wide range of materials can be tailored for discrete applications.

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