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Review Article

Scaffolds Based Bone Tissue Engineering: The Role of Chitosan

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As life expectancy increases, malfunction or loss of tissue caused by injury or disease leads to reduced quality of life in many patients at significant socioeconomic cost. Even though major progress has been made in the field of bone tissue engineering, present therapies, such as bone grafts, still have limitations. Current research on biodegradable polymers is emerging, combining these structures with osteogenic cells, as an alternative to autologous bone grafts. Different types of biodegradable materials have been proposed for the preparation of three-dimensional porous scaffolds for bone tissue engineering. Among them, natural polymers are one of the most attractive options, mainly due to their similarities with extracellular matrix, chemical versatility, good biological performance, and inherent cellular interactions. In this review, special attention is given to chitosan as a biomaterial for bone tissue engineering applications. An extensive literature survey was performed on the preparation of chitosan scaffolds and their *in vitro* biological performance as well as their potential to facilitate *in vivo* bone regeneration. The present review also aims to offer the reader a general overview of all components needed to engineer new bone tissue. It gives a brief background on bone biology, followed by an explanation of all components in bone tissue engineering, as well as describing different tissue engineering strategies. Moreover, also discussed are the typical models used to evaluate *in vitro* functionality of a tissue-engineered construct and *in vivo* models to assess the potential to regenerate bone tissue are discussed.

Introduction

Bone tissue, when injured, leads to dramatic changes in the quality of life of patients. It can limit the ability to perform basic tasks, such as walking and frequently causes social and psychological problems. The current clinically available solutions for these problems rely on bone graft transplants (autologous, allogeneic, and xenogenic), bone transport methods (Ilizarov technique), and implants based on different types of materials. More than 2.2 million bone graft procedures (autologous bone graft and banked bone) take place annually worldwide.[1,2] Those procedures ensure adequate bone healing of many skeletal problems, such as nonunion fractures, cervical and lumbar spine fusion, joint arthrodesis, or revision arthroplasty. Bone grafting is a strong and mature business generating sales of more than $2.5 billion a year.[3] Autografts are considered the gold standard for bone repair. However, some complications may occur, such as bone nonunions and blood loss, which increases the need for blood transfusions.[4–6] Moreover, besides being an expensive procedure, there is a limited supply of tissue and it causes significant donor-site morbidity.[7,8] Allografts are typically nonvital (dead) bone harvested from a cadaver and then processed using a freeze-drying method that extracts all the water via a vacuum drying. These types of grafts avoid donor-site morbidity but present a potential risk for disease transmission and severe immune response by the patient.[9] Similar to allogeneic bone, xenogenic bone is nonvital bone derived from other species, mainly from bovine origin. Because the potential for immune rejection and contamination by viral proteins is higher in bovine bone than in human cadaver bone, xenograft material is processed at very high temperatures. The Ilizarov methodology consists of an osteotomy followed by bone distraction by extendable fixation devices. This technique avoids problems related with the osseointegration of bone grafts, but requires longer periods of treatment (12–18 months) and can be quite painful for the patient.[10] The aforementioned limitations justify the development of new therapies using alternative concepts that are currently the focus of intense research efforts.

Bone has a notable regenerative ability but a considerable amount of bone loss or the development of an adverse microenvironment can hinder this capacity, such as in cases of severe trauma, developmental deformities, revision surgeries, and tumor resection.[11,12] In these cases, bone tissue engineering holds the promise of great therapeutic potential.[13]

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Bone tissue engineering may constitute the needed breakthrough technology to solve the problem of bone shortage in various destructive clinical conditions and deformities by providing functional tissue-engineered biological substitutes.[14] The most promising strategy used in this field is based on the seeding and *in vitro* culture of primary osteoblasts or adult stem cells (ASCs) differentiated into the osteogenic phenotype on three-dimensional (3D) scaffolds (synthetic, natural, or ceramic). These constructs would be subsequently implanted into a bone defect. The cells would synthesize the extracellular matrix (ECM) of new bone tissue, whereas the scaffold would provide an adequate 3D environment for the cells to adhere, proliferate, and differentiate. The scaffolds will not only be temporary 3D support for cells to create new bone, but also space filling and controlled local release device of signaling molecules. To accomplish all of these goals, scaffolds should meet stringent requirements, such as biodegradability at a rate that is compatible with the rate of new tissue formation. Other important properties include biocompatibility with host tissues, nontoxicity and nonimmunogenicity, appropriate mechanical properties, adequate porosity, and morphology.[15–17] All of these properties are essential to facilitate and guide cell ingrowth and transport of gases, metabolites, nutrients, and signaling molecules, both within the scaffold and between the scaffold and the native local environment.

The selection of the most suitable material to produce a scaffold to be used in bone tissue engineering applications is a determinant step, since its properties will determine its final characteristics. Biodegradable polymers, either synthetic or natural, are the most appropriate substrates for cells to attach, grow on, and maintain a differentiated phenotype. In recent years, naturally derived polymers have been increasingly proposed for the referred application. In our group, we have been working with natural polymers, such as in starch,[18–22] chitosan,[23–27] gellan gum,[28–31] soy,[32] and silk.[33,34] Our strategy is to mimic nature and for that we have been using these polymers to design functional microenvironments stimulating tissue morphogenesis. In particular, chitosan has shown an excellent combination of properties and it has been demonstrated that it is a suitable biomaterial for the development of scaffolds for bone tissue engineering. Chitosan can be used either alone[23,35–41] or in combination with other biodegradable polymers, such as aliphatic polyesters,[25,27,42–45] other natural polymers such as starch[26,46,47] or silk,[48,49] or with ceramics such as hydroxyapatite (HA).[24,50–58]

This article aims to provide an overview of the most important concepts in bone tissue engineering and a review of chitosan-based scaffolds proposed to use in bone regeneration. The potential of this biomaterial as a suitable substrate to support osteogenic differentiation of mesenchymal stem cells (MSCs) will also be explored.

Brief Overview of Bone Biology

Bone is a dynamic and complex tissue evolving and adapting to various stimuli throughout one's lifetime.[59] It plays crucial roles in both mechanical support and mineral homeostasis.[60] Within a skeletal element, there are different morphologies of bone, such as cortical and trabecular bone. Cortical bone is a compact structural tissue, with only 10% porosity, being 80% of the mass of an adult human skeleton. Trabecular bone is a spongy structure with 50%–90% porosity, filled with bone marrow. The majority of bones are covered by a highly vascularized fibrous connective tissue, the periosteum.[61] Five different cell types are involved in bone maintenance and remodeling: MSCs, bone-lining cells, osteoblasts, osteocytes, and osteoclasts. Within the bone structure, MSCs are found in the bone marrow[62–65] and also in the periosteum.[66] Bone marrow is composed of hematopoietic tissue and the supporting stroma.[67] Marrow stromal cells, originally thought to only contribute to the hematopoietic microenvironment, later came to the center stage with the recognition of being the stem/progenitor cells of skeletal tissues.[63] Human autologous bone marrow associated with macroporous HA scaffolds was implanted in large bone segmental defects and shown to promote bone regeneration.[68] After a 7-year follow-up,[69] patients presented a complete healing of their defects. Bone-lining cells are flat cells that cover all bone surfaces and are believed to arise from osteoblasts that become inactive.[70,71] These cells form an important cellular barrier that divides the canalicular network (where osteocytes are present) from other fluids.[61] Osteoblasts can be derived from MSCs that synthesize the osteoid (nonmineralized organic matrix of the bone, that is, type I collagen, osteocalcin, osteopontin, bone sialoproteins, and bone morphogenetic proteins).[72] Osteoblasts also have an active role in the vascularization process by secreting morphogens that activate angiogenesis by signaling endothelial cells.[73–75] Osteocytes are terminally differentiated osteoblasts entrapped within the bone ECM that are involved in the maintenance of ECM and calcium homeostasis.[61] Osteocytes also sense mechanical stress and communicate signals for bone remodeling and tissue maintenance.[76] The fifth cell type is the osteoclast, responsible for bone resorption, which is the first stage of the bone remodeling process, followed by bone homeostasis. These cells are large multinucleated cells differentiated from a fraction of monocytes found in peripheral blood.[61]

As with many other connective tissues, one of the main components of bone is its ECM, which in this case is mineralized. Bone ECM is composed of 35% organic matrix and 65% mineral matrix. The most abundant mineral in bone ECM is HA, a calcium phosphate crystallized at the surface of collagen fibrils, required to resist bending and compression stresses.[59] The organic matrix is mainly protein composed of type I collagen (90%) and the remaining fraction includes up to 200 other noncollagenous proteins, such as glycoproteins, proteoglycans, integrin-binding proteins, and growth factors.[59]

Bones are developed by two main processes: intramembranous and endochondral ossification.[77,78] Intramembranous ossification is a process that generates flat bones and the skull structure. In this pathway, the embryonic mesenchyme condenses and develops in primary ossification centers, which will eventually fuse to form a network of anastomosing interconnected trabeculae made of woven bone.[77] After that, periosteum is formed at the surface of trabeculae, further mineralized and part of the intertrabecular connective tissue is transformed in hematopoietic tissue.[78] Finally, the woven bone is remodeled into a lamellar type of bone.[79] Endochondral ossification is an osteogenic process through which long bones, vertebrae and the pelvis are

generated from precursor cartilaginous tissue.[80] This process starts in the fetus where MSCs differentiate into chondrocytes, converting the condensed mesenchyme into a cartilaginous model of bone that will expand in its extremities, while becoming hypertrophic in the center. These hypertrophic chondrocytes will promote primary ossification by secreting molecules (such as alkaline phosphatase [ALP], type X collagen, or vascular endothelial growth factor) that will induce calcification of cartilage. This tissue will be resorbed, becoming a structure onto which progenitor cells differentiate into osteoblasts that start to deposit osteoid. After birth, secondary ossification centers develop at the extremity of long bones, allowing the development and growth of bone structure.[79]

Bone Tissue Engineering Strategies

Bone has an intrinsic self-ability to regenerate, but over a large defect, inherent osseous processes are not able to repair the defect during the patient's lifetime.[81] Further, diseased bones do not heal properly and, under certain pathological conditions, start damaging themselves.[81–83] Tissue engineering has emerged as a possible solution for these clinical conditions. Several strategies can be employed to develop new bone tissue. Those strategies may involve the use of an ECM-like structure (scaffold), cells, and/or growth factors. These three basic components need to be well synchronized to achieve a successful tissue engineering therapy. The strategy used for a specific bone defect must be adapted to the clinical state of the patient. Overall, there are primarily three approaches that have been described for tissue engineering strategies: (1) to use engineered on matrices alone, to guide tissue regeneration; (2) to inject autologous, allogeneic, or xenogeneic cells alone; (3) to develop constructs of cells seeded on these matrices.[14] The first method involves implanting the scaffold at the site of interest, allowing host cells to migrate from the surrounding tissues to colonize the scaffold. The second strategy has the advantage of involving minimal surgical invasion and cells can be manipulated by recombinant gene technology or clonal expansion before injection or infusion. However, this methodology has limitations for bone critical-sized defects, due to the absence of the supporting matrix to keep cells at the defect site. In the last approach, cells are seeded onto the scaffold (construct) and later implanted into the bone defect. Usually, constructs are produced *ex vivo* before transplantation to a bone defect and over time seeded and/or host cells will synthesize a new ECM, as the scaffold degrades, creating a new functional tissue. This review will focus on the third tissue engineering strategy, exploring the potential use of autologous stem cells cultured in biodegradable scaffolds that will act as ECMs, supporting cell growth and tissue development.

Use of Naturally Occurring Polymers in Scaffolds

Nature offers a remarkable set of materials with great potential to be used in different fields. The study and use of natural materials comes from ancient times, such as cellulose, which is used to produce paper or silk to produce clothes. In medicine, for example, chitosan is used as a wound-dressing material and collagen as a substitute in reconstructive surgery. Today, powerful tools are available and the micro- and nanostructures of these materials have been described. This new level of knowledge has opened new opportunities to develop materials for other applications, such as scaffolds for tissue engineering. Great efforts have been made to recapitulate the key features of bone ECM by developing structures that mimic this naturally occurring matrix. ECM plays an important role in cell activities, modulating their behavior.[84] One difficulty in developing such scaffolds is the complexity of recreating microenvironments similar to that found in the tissue of interest. A simple approach to mimic nature is to use naturally occurring materials. Moreover, natural polymers have different functions, such as the role of polysaccharides in cell membranes, intracellular communication and storage, or proteins that are structural materials and catalysts (enzymes).[85] Natural polymers such as starch[18–20,22,86–88] or chitosan[89–92] have been described as biocompatible, biodegradable, and having tailorable degradation rates.[86,90] Some drawbacks of these biomaterials are the limited mechanical properties and processability or variability between different batches.[15] Examples of natural polymers commonly used to produce scaffolds are collagen,[93–97] hyaluronan,[98,99] alginate,[100] silk,[48,101,102] and chitosan.[23,37,41] These polymers can be combined with other synthetic materials, to improve their processability and mechanical properties. Combinations with HA,[103] aliphatic polyesters,[25,27,104,105] or composites of different natural polymers[26,46] have also been described. Herein a special focus will be given to the natural polysaccharide chitosan, the deacetylated product of chitin obtained from the exoskeleton of crustaceans.

Chitosan as a Natural Origin Biopolymer

The history of chitosan dates back from the 19th century when Rouget discussed its deacetylated form.[106] Chitosan is a linear polysaccharide, obtained from the deacetylation of chitin, the primary structural polymer of the exoskeleton of crustaceans, cuticles of insects, and cell wall of fungi.[107,108] Chitosan is composed of glucosamine and *N*-acetyl glucosamine with β (1–4) link.[109] Chitosan is the common name for the family of deacetylated chitins, with different degrees of deacetylation. By definition, when the number of *N*-acetyl glucosamine units is higher than 50%, the polymer is considered chitin. On the other hand, when the number of *N*-glucosamine units is superior, its name is chitosan.[110] The molecular weight of chitosan may range from 300 to more than 1000 kDa, depending on its origin and preparation method.[35] Chitosan is a semi-crystalline polymer and its crystallinity is dependent of the degree of deacetylation.[107] The solubility of chitosan depends on the free amino and *N*-acetyl groups, which are soluble in acidic pH.[35] The cationic nature of chitosan allows electrostatic interactions with anionic glycosaminoglycans (GAGs) and proteoglycans. Natural polymers are known to influence cell morphology, modulation, and differentiation,[111,112] as referred to previously. This property is of crucial importance in the tissue engineering field, because GAG molecules modulate the action of several cytokines and growth factors.[113]

Chitosan presents a wide range of properties that make it appropriate for tissue engineering applications, namely, its biodegradability,[89,114,115] biocompatibility,[91,92,116] antibacterial activity,[117–119] wound healing properties,[120–123] and bioadhesive character.[124]

Chitosan can be hydrolyzed by chitosanases,[125] which are absent in mammals. Lysozyme is responsible for the

biodegradation of chitosan *in vitro*.[89,126] The degradation rate of chitosan is inversely related to the degree of deacetylation,[127] which represents the proportion of *N*-acetyl-D-glucosamine units to the total number of units.[125] Lysozyme is ubiquitous in the human body.[128] It is found in the lacrimal gland, middle ear, nose, bronchus, bronchiole, bone marrow, and digestive tract.[129] Lysozyme has an important role in inflammatory response, being secreted by macrophages, monocytes, and granulocytes.[130,131] Monocytes and macrophages are the main contributors to the presence of lysozyme in human serum in concentrations between 7 and 13 mg/L.[128]

Chitosan has intrinsic anti-microbial properties against several microorganisms, namely, fungi and bacteria.[132] The mechanism that results in these properties is unknown, although its cationic nature associates with anions in bacterial cell walls, suppressing biosynthesis and also disrupting the mass transport across the cell wall, leading to the death of bacteria.[132]

Chitosan has been described as a potent wound-healing accelerator,[133] and to possess immunological activity, by activating macrophages,[134] to produce cytokines[135] and to inhibit infection.[136]

One of the most important characteristics of chitosan, for tissue engineering applications, is its ability to be shaped into various structures, such as microspheres,[137] paste,[138] membranes,[111] sponges,[37,139–142] fibers,[27,38,143] and porous scaffolds.[25,27,54,144,145] Several processing methodologies have been used to produce porous chitosan scaffolds and will be discussed in detail in this article. Nevertheless, before describing scaffold processing techniques, it is important to underline the properties that a scaffold must possess to be successfully applied in bone tissue engineering applications.

Scaffold Requirements for Bone Tissue Engineering

Bone is a 3D tissue and cells alone do not grow in a 3D manner *in vitro*. For that reason, a tridimensional structure is required to support the formation of new functional bone tissue. This structure should provide a suitable environment for cell attachment, proliferation, differentiation, and ECM deposition. The *in vitro* cultured constructs, when implanted into the defect, must be vascularized and osteointegrated into the host bone.[146] The 3D structures should be biocompatible, that is, not evoking an immune response when implanted in the host tissue. When a scaffold is implanted into the defect to restore bone functionality, it should activate the healing mechanisms (inflammatory response). The time course of healing is influenced by interactions between blood, scaffold surface, and degradation products, which are released from the scaffold and therefore, influencing biocompatibility. The ideal scaffold should degrade at a rate compatible with the rate of bone growth, physically creating open space for new bone formation, until full regeneration is achieved. The process of polymer degradation follows the mechanisms through which polymer chains are cleaved into oligomers and finally to monomers, which can be metabolized by natural mechanisms.[147] If a biological process mediates the degradation process, it is designated as biodegradation.[147] Several factors influence the kinetics of degradation: type of chemical bonds, pH, polymer composition, crystallinity, molecular weight, porosity, water uptake, and anatomical location of the implant.[147] Ideally, natural pathways of the animal body should eliminate the degradation products.

As previously discussed, bone is a highly vascularized tissue relying on the interactions between bone cells and blood vessels. In this way, angiogenesis and neovascularization play a crucial role in bone repair, and should be taken into account when designing a scaffold. Angiogenesis is mainly characterized by the protrusion and outgrowth of capillary buds and sprouts from pre-existing blood vessels, whereas neovascularization comprises the formation of functional microvascular networks with red blood cell perfusion.[148] Both processes are required to ensure successful engraftment of the construct into the surrounding host tissue. A vascular network can be included in a biodegradable and biocompatible scaffold by microfabrication techniques.[149] The main property of the scaffold that is directly related to vascularization is its porosity.[150] Scaffolds should have highly interconnected pores to promote cell ingrowth and distribution throughout the matrix, as well as facilitating the development of neovascularization. The minimum pore size is considered to be approximately 100–150 μm,[151] due to cell size, migration requirements, and fluid transport. However, due to vascularization requirement, pore sizes were shown to affect the course of osteogenesis.[150] Large pores rapidly become well-vascularized, leading to direct osteogenesis.[152,153] In contrast, small pores lead to hypoxic conditions, which tend to induce the development of an osteochondral process, before osteogenesis occurs. The porosity strongly influences scaffold mechanical properties. High porosity and pore size facilitates tissue ingrowth, but the consequence is a drastic reduction of mechanical properties, compromising the structural integrity of the scaffold.[154] The mechanical properties of a scaffold should be compatible with those of the native tissue, maintaining its structural integrity after implantation.[155] In general, the scaffold should be strong enough to not only resist stresses caused by the surrounding environment that may cause important dimensional changes. Scaffold integrity is critical, since cells and tissue remodeling are important to achieve a stable biomechanical environment and vascularization at the host site. The topography and surface chemistry of the scaffold play a crucial role in its performance, since those are the first elements that cells recognize when in contact with the scaffold surface. The hydrophilicity and hydrophobicity of the scaffold surface will modulate protein adsorption, which will in turn influence cell seeding.[156]

The methodology used to produce scaffolds for bone tissue engineering must not adversely affect biocompatibility or physical and chemical properties of the biomaterials used. Different scaffold batches may exhibit minor variations in their properties when prepared using the same parameters and conditions.[157] Different processing methodologies for chitosan-based scaffolds have been already reported and will be further discussed in detail.

Chitosan Scaffold Production Methods

The most common methodology for producing chitosan scaffolds utilizes freeze-drying. This process consists of the lyophilization of a frozen chitosan solution, where the chitosan acetate salt is induced by the freezing conditions to phase-separate from the ice crystal phase. The ice phase is

further sublimated, producing a porous structure.[24,35,37,49,54,142,145,154,158–169] In most cases, the scaffolds can still have chitosan acetate that will cause fast swelling and subsequently dissolution in a neutral aqueous medium. This can be overcome by cross-linking upon immersion in sodium hydroxide,[35,163] sodium sulfate,[38] tripolyphosphate,[37,142] ethanol series,[35] or with a combination of cross-linking with rehydration.[158] The freeze-drying technique requires very tight control of the temperature. If the temperature is not sufficiently low, the matrix will not become rigid enough to support the interfacial tension caused by the evaporation of the solvent without collapsing, creating a surface skin. Another limitation of the structures produced by this technique is that pore size is not very large. Also, the mechanical properties of the porous structures are very limited, even after cross-linking. Due to these limitations of freeze-drying, solvent-exchange/phase-separation has been proposed based on the gelation of a solution of chitosan using an alkaline solution below its gelation point.[26,36,51,170] In a freeze-drying process the choice of the solvent is limited, since the solvent vapor pressure at the drying temperature (usually low) must be high enough to allow its removal.[36] With this alternative method, which is also less time consuming and more economic, the choice of the solvent is wider.[36]

Another processing methodology for chitosan is wet spinning, which allows the production of fibers. Due to the strong inter-chain forces derived from the hydroxyl and amino groups, chitosan tends to degrade at temperatures below its melting temperature, limiting its processability by melt or dry spinning methods.[38,41,143,171–173] Basically, chitosan is dissolved in a solution of diluted acetic acid. This solution is spun through a spinneret into a coagulation bath, in this way producing fibers. Chitosan can also be processed by electrospinning into a nanofiber mesh scaffold. This method uses an electrical field created between a collector and a capillary connected to a reservoir with the polymer solution. The elongation of the drop of solution caused by the electrical field leads to the formation of very thin fibers with nanometer scale diameters. Electrospinning of pure chitosan[39,116,174–176] is considered to be quite difficult, since the resulting chitosan salt is soluble in water. Its stability in solution requires neutralization or cross-linking in a post-processing stage that frequently has an impact on the morphology of the mesh. Several studies report the blending of chitosan with other polymers, being easier to process by electrospinning, namely silk fibroin,[177] poly(ethylene oxide),[178,179] poly(vinyl alcohol),[180] collagen,[181] or polycaprolactone.[182]

Less conventional is the particle aggregation method (Fig. 1) proposed by Malafaya *et al.*[23] This process relies on the bioadhesive character of chitosan that confers a strong bonding between individual particles. The scaffolds produced by this method have shown very interesting mechanical properties. In another study, chitosan-poly(lactic-co-glycolic acid; PLAGA) microspheres were molded by mixing them with acetic acid solution in a stainless steel mold.[183]

Rapid prototyping may be another processing route enabling the manufacture of porous chitosan scaffolds. This methodology is based on the production of a 3D physical model from computer-aided design data (CAD software), which is generated in a layer-by-layer deposition process.[144,184] Theoretically, a great variety of morphologies and shapes can be created by different variants of this technique resulting in reproducible geometry and mechanical properties.

Our group developed different chitosan-based scaffolds by melt-based routes.[43,45] The vast majority of the processing methods used to produce chitosan scaffolds involve the use of solvents. Resident solvents are frequently toxic if they are entrapped be entrapped in a scaffold. We have developed various blends of chitosan with different aliphatic polyesters. Those blends can be processed by compression molding followed by salt leaching,[43] melt spinning, and fiber bonding[27,45] into porous scaffolds with different morphologies and mechanical properties (Fig. 2).

A systematic list of various porous scaffold compositions using chitosan, the processing methods used to obtain scaffolds, and the *in vitro* evaluation with different cell types is provided in Table 1. It is clear from this table that the most used processing method to obtain chitosan-based porous scaffolds is freeze-drying or another freeze-related process. The main reason for this observation may be the simplicity of the process.

In Vitro Cellular Approach in Bone Tissue Engineering

The development of new scaffolds follows a typical evaluation routine. The first step is the assessment of potential cytotoxicity. This initial screening is based on the use of extracted leachables from the scaffold, that is, substances that

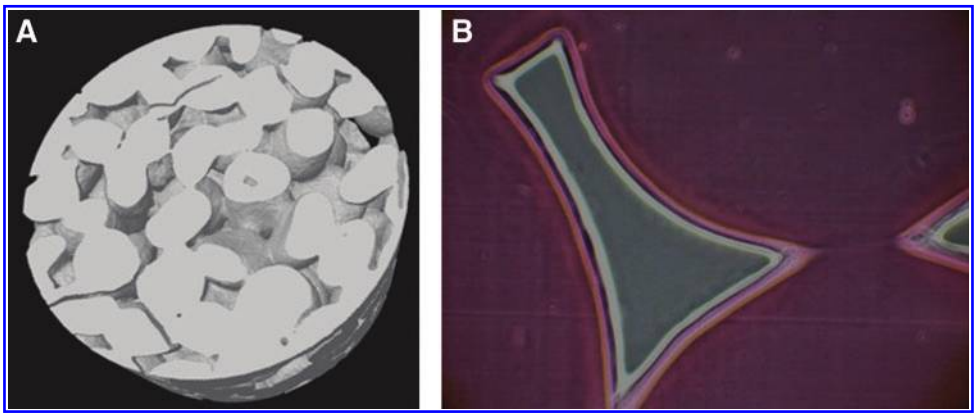

FIG. 1. Microcomputed tomography image of a cross section of chitosan scaffolds obtained by the particle aggregation method **(A)** and interface between the chitosan particles stained with eosin **(B)**. Color images available online at www.liebertonline.com/teb

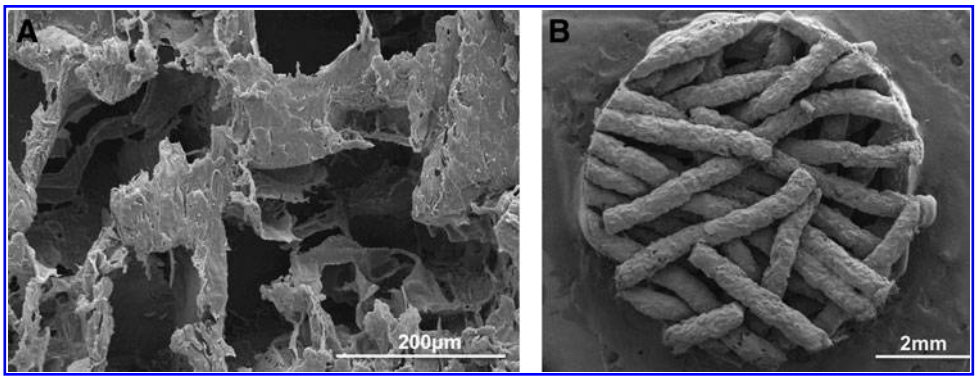

FIG. 2. Chitosan-based scaffolds produced by compression molding followed by particle leaching **(A)** and fiber bonding **(B)** methodologies.

leach out of the biomaterials. These leachables are added in defined concentrations to standard culture medium and placed in contact with a cell line for a determined period. After this, cell viability and cell morphology are evaluated to determine the eventual toxicity to the cells. The use of cell lines is recommended in first stage testing, given that these cells are reproducible and can be expanded to large numbers. Cell lines, such as mouse fibroblast cells (L929) or human osteosarcoma cells (SAOS-2), are frequently used. If the scaffolds show no signs of cell cytotoxicity or morphology changes, the next step involves direct contact assays with an appropriate cell type to evaluate the cytocompatibility and phenotype functionality. A valid 3D construct for bone tissue engineering applications should have a positive outcome from this sequence of initial *in vitro* tests.

Selection of cells

Ideally, the cell source for scaffolds seeding should be nonimmunogenic, easily available, nontumorogenic, and with other defined and adequate characteristics. It should be expandable into large numbers and have demonstrated osteogenic potential. Autologous cells, from each patient, are preferred.[185–189] These cells may be isolated from the biopsy of a patient's tissue (e.g., cartilage, bone, and skin) from the patient. The tissue obtained is dissociated and the isolated cells are expanded in culture for later implantation into the same patient.[29,68,185] The use of autologous cells eliminates the risk of immune rejection and the need to use immunosuppressive drugs. For bone tissue engineering applications, osteoblasts are the most obvious selection, since those cells are responsible for the bone formation.[72] However, these cells may have limited availability since the number of cells that are obtained after the isolation procedure is low and the expansion rate may be slow.[190] In recent years stem cells have been studied as a viable alternative to isolated autologous cells.[190] The term "stem cell" is used to describe undifferentiated cells with an ability to self-renew while maintaining the capacity for multi-lineage differentiation.[63] There are primarily two types of stem cells that have been studied for tissue engineering applications, embryonic or adult. Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of a blastocyst stage embryo.[191] These cells possess long-term proliferation potential and are able to differentiate into all of the types of somatic cells in the organism. Ethical issues have been raised regarding the source of ESCs, which may limit their use in regenerative medicine. ASCs may be a valid alternative to ESCs in many applications. These cells can be isolated from different adult tissue sources such as bone marrow,[63] peripheral blood,[192] adipose tissue,[193] or fetal tissues such as umbilical cord,[194] amniotic fluid,[195] or placenta.[196] ASCs are multipotential cells capable of differentiating into several cell lineages such as osteoblasts, chondrocytes, and adipocytes.[197] Recent reports sustain even a plasticity of ASCs, that is, their ability to be differentiated into other types of cells.[198]

The process of osteogenic differentiation of stem cells may be achieved by expanding the cells in standard culture medium, supplemented with β-glycerophosphate,[199] ascorbic acid,[200] and dexamethasone.[65,200] These agents activate the osteogenic commitment of ASCs. Culture of osteogenic cells depends on the adequate supplementation of their growth medium with a source of inorganic phosphate,[201] β-glycerophosphate, a nonphysiological organic substrate of ALP,[202] to produce mineralized ECM. Ascorbic acid is essential for the survival of human osteoblasts *in vitro*.[203] This osteogenic inducing agent is required for collagen synthesis and ALP activity.[200] Dexamethasone is a glucocorticoid that increases the expression of several genes associated with osteogenic differentiation.[204] Moreover, the timing, size, and number of bone-like nodules is affected by the dose of dexamethasone used.[205] Osteogenic medium can also be supplemented with growth factors that naturally occur in bone, such as bone morphogenic proteins (BMPs), fibroblast growth factors, platelet-derived growth factor (PDGF), transforming growth factor beta, and insulin growth factors.[206–213] The process of osteogenic differentiation is coordinated and involves three main stages: (i) cell proliferation; (ii) ECM deposition and maturation; and (iii) mineralization.[214] During cell proliferation, growth genes are expressed.[215] Immediately after the downregulation of proliferation, the expression of ALP increases.[214] During this period, the ECM undergoes a series of events that renders it competent for mineralization (ECM maturation and HA formation).[215] After this stage, ECM becomes mineralized.[214,215] With the onset of mineralization, the ECM protein genes become upregulated; for example, osteopontin and osteocalcin are increasingly expressed with the accumulation of mineralization.[214] ALP activity before the onset of the mineralization suggests that this enzyme is involved in the preparation of ECM for mineral deposition.[214] This enzyme is considered to be an early marker of osteogenic differentiation[20,216,217] and used as an *in vitro* assessment of osteogenic differentiation. The mineral content of the bone ECM can be qualitatively assessed by alizarin red

Table 1. Survey of *In Vitro* Studies with Chitosan-Based Scaffolds Proposed in the Literature for Bone Tissue Engineering Applications

Scaffold structure	*Processing method*	*Cell type (source)*	*References*
Chitosan scaffolds	Freeze-drying	—	35
Chitosan-TCP sponges	Freeze-drying	Fetal rat calvaria cells	141
Chitosan-gelatin scaffolds	Freeze-drying	—	157
Chitosan-TCP sponges	Freeze-drying	MG63 human cell line	184
Chitosan-HA scaffolds	RP and freeze-drying	—	143
Chitosan-calcium phosphate scaffolds	Freeze-drying	MG63 human cell line	54
Chitosan scaffolds	Freeze gelation	ROS 17/2.8 cells	36
Chitosan sponges	Freeze-drying	Rat calvaria cells	37
Chitosan fiber mesh scaffolds	Wet spinning	Human SAOS-2 cell line	38
Chitosan scaffolds	Freeze-drying	MG63 human cell line	158
Chitosan-silk scaffold	Freeze-drying	—	49
Chitosan scaffolds	RP	Porcine BMSCs	183
Chitosan scaffolds	Electrospinning	—	39
Chitosan-gelatin scaffolds	Freeze-drying	HUVECs	185
Chitosan scaffolds	Precipitation/Particle aggregation	ADAS	23
Chitosan sponges	Freeze-drying	MG63 human cell line	160
CPC-Chitosan scaffold	Cement/Particle leaching	MG63 human cell line	186
Chitosan-starch scaffolds	Solvent-exchange phase separation	—	46
Chitosan scaffolds with HA formation	Freeze-drying	Human SAOS-2 cell line	162
Chitosan-nanoHA scaffolds	Freeze-drying	MC3T3-E1 cell line	161
Chitosan-coralline scaffolds	Freeze-drying	CRL-12424 cell line	163
HA-chitosan scaffold	Freeze-drying	Goat bone marrow cells	24
Chitosan-gelatin scaffolds	Freeze gelation	Human BMSCs	51
BCP-chitosan scaffolds	Freeze-drying	MC3T3-E1 cell line	164
Chitosan-PLAGA scaffolds	Particle aggregation	MC3T3-E1 cell line	42
Chitosan gelatin/montmorillonite scaffolds	Freeze-drying	Rat stromal cells TC1	165
Chitosan scaffolds	Freeze gelation	—	40
Chitosan and chitosan-starch scaffolds	Freeze gelation	Human SAOS-2 cell line	26
Chitosan-collagen sponges	Freeze-drying	Rat BMSCs	166
Chitosan-PBS/PBTA/PCL	Compression molding/salt leaching	Mouse BMC-9 cell line	25
Chitosan scaffolds	Wet spinning	Mouse osteoblast 7F2 cell line	41
Chitosan-PBS scaffolds	Melt spinning/fiber bonding	Human BMSCs	27
Chitosan-PBS/PCL/PBTA/PBSA	Compression molding/salt leaching	—	43
Chitosan-PCL scaffolds	Electrospinning	MC3T3-E1 cell line	181
Chitosan scaffolds	Freeze-drying	MC3T3-E1 cell line	168
PLGA-chitosan scaffolds	Freeze-drying	Human BMSCs	167
Chitosan sponges	Freeze-drying	Chicken embryo chondrocytes	187
Chitosan and chitosan-starch + lysozyme scaffolds	Freeze gelation	Rat BMSCs	188
PCL-chitosan	Solvent casting/salt leaching/ freeze drying	Rat osteoblasts	44

HA, hydroxyapatite; TCP, tricalcium phosphate; RP, rapid prototyping; PLLA, poly(L-lactic acid); ROS, rat ostosarcoma cells; HUVECs, human umbilical vein endothelial cells; ADAS, adipose-derived stem cells; CPC, calcium phosphate cement; BCP, biphasic calcium phosphate; PLAGA, poly(lactic-co-acid-glycolic acid); PBS, poly(butylene succinate); PCL, polycaprolactone; PBTA, poly (butylene terephtalate adipate); PBSA, poly(butylene succinate adipate); PLGA, poly(L-glycolic acid); BMSCs, bone marrow mesenchymal stem cells.

or von Kossa staining, and the calcium content can be quantitatively assessed. This information can be complemented by the analysis of the mineral fraction by energy dispersive spectroscopy, which detects the presence of calcium and phosphorous elements. Thin-film X-ray diffraction may be used to analyze the crystallinity of the ECM. Fourier-transformed spectroscopy may be used to detect carbonate and phosphate groups.[27,218]

In vitro *studies with chitosan as a biomaterial*

It is well accepted that cells strongly interact with their environment, namely, with neighboring cells, ECM, and the surface to which they adhere.[219] Chitosan as a biomaterial as previously mentioned has an analogous structure to the GAGs present in connective tissue ECM. Several studies describe the positive influence of chitosan on cell attachment, proliferation, and the osteogenic differentiation of MSCs (Table 1). Mouse MSCs in contact with a chitosan suspension were shown to improve osteogenic differentiation, when compared to cells seeded onto polystyrene culture wells.[220] Lahiji *et al.* reported that chitosan-coated coverslips are an appropriate substrate for the growth of human osteoblasts and chondrocytes.[108] Poly(D,L-lactic acid) films modified with chitosan solution improved cell adhesion, proliferation, and biosynthetic activity, using human osteoblasts.[221] Moreover, neonatal rat calvaria osteoblasts proliferate at superior rates on titanium surfaces coated with chitosan compared with titanium alone.[222] In fact,

coating of titanium pins with chitosan induced minimal inflammatory response and a positive healing of a rabbit tibial defect.[223] MC3T3-E1 osteoblast-like cells proliferated and increased ALP activity, as well as upregulation of osteogenic gene expression, in composite chitosan/PLAGA scaffolds as compared to PLAGA scaffolds.[183] Chitosan–collagen sponges with higher concentration of chitosan positively promoted osteoblastic differentiation of bone marrow mesenchymal stem cells (BMSCs) and improved the mechanical and physical properties of the matrices.[167] Previous studies from our group, using flat discs obtained by injection molding composed of chitosan-poly(butylene succinate) (PBS) and PBS blends, showed that chitosan had a positive effect on osteoblast-like cells.[224] Further, two groups reported that PCL nanofibrous scaffolds containing chitosan revealed that stem cells adhered, proliferated, and expressed phenotypic markers of osteogenic differentiation in a superior way compared to nanofibrous scaffolds alone.[182,225] The ability of chitosan to support cell adhesion and influence osteogenic differentiation of cells can be attributed to its chemical properties.

In vitro testing systems are inevitably limited in their capacity to recreate the complex *in vivo* environment. Therefore, these tests may be unable to accurately predict *in vivo* performance, particularly in the context of tissue engineering and regeneration of functional tissues. Thus, in later stages of the development of a tissue engineering strategy, it may be critical to include *in vivo* experiments.

In Vivo Animal Models

The general trend in bone tissue engineering after successful *in vitro* testing of the constructs is to implant the *in vitro* tissue-engineered construct in a relevant animal model. Often, an ectopic small animal model (e.g., mouse or rat) is first. Constructs are implanted into a nonbone anatomic location of the animal's body.[88,226–228] Those areas can be intraperitoneal, intramuscular, mesenteric, or subcutaneous. These models are also useful in determining whether a scaffold has adequate properties, for example, sufficient porosity and pore interconnectivity to allow tissue ingrowth and neovascularization. It is important to determine the biodegradation of implant materials, in terms of both degradation products and the host immune response. If the aim is to use human cells, nude mouse/rat models are commonly used. These models can be used to screen tissue-engineered constructs for osteoconductivity (i.e., the ability of the scaffold to induce proliferation of undifferentiated stem cells), the differentiation of seeded cells to an osteogenic lineage, and the formation of ectopic bone.[229–232]

An *in vivo* approach should mimic the clinical situation closely as possible. Some models use an intraosseous wound that will not heal spontaneously (i.e., critical-sized defect).[81] The minimum size of a critical-sized defect is not absolutely clear. This is a complex situation as a defect cannot be defined only by size; it is dependent on other variables (e.g., other species and anatomic location).[233] Guidelines are available for the dimensions of implants based on the size of the animal, bone chosen, and an implant design that avoids pathological fracture.[234] It is important to include controls in the experimental design. These controls should be of a material already in clinical use and also a control consisting of an empty defect, to prove that the bone defect is not able to regenerate itself.[234] There are several types of bone defects that can be used, such as cranial, segmental, partially cortical, and cancellous bone. These locations can be subjected to load or nonload bearing (e.g., femur or calvarial, respectively). The type of animal can be small (mouse or rat)[88,102,213,235,236] or large (rabbit, sheep, goat, dog, or nonhuman primates).[140,185,237–242] Typically, researchers start with a smaller model that is less costly and where the experimental results may be easy to compare with many other experiments reported in the literature. One of the most accepted nonloading bearing models is the calvaria bone defect. This flat bone allows the creation of a uniform circular defect, and has sufficient size to make easy surgical procedure and handling in small animals. In those models, the dura mater and the overlying skin provide fixation of the scaffold. The model has been systematically studied and is very well established.[81,82,243,244] This model can be performed in small animals using rat[245,246] or mouse.[213,235] It can be also applied to large animals, like rabbit[247–249] or sheep.[189]

The last stage of preclinical trials of a bone tissue engineering strategy should be performed in animals that are believed to be more similar to humans, in terms of metabolism, physiology, anatomy, etc. Bones of small animals are more reactive to specific stimuli and are not subjected to comparable stresses. For example, a femur defect in rats[250–252] is believed to heal faster that in larger animals.[243] However, in a study where the authors compared the bone ingrowth using the same chamber, in rats and in goats, no significant differences were observed between the two animals.[253] *In vivo* experimental design is therefore not an easy task. It is necessary to balance all the variables and decide which animal model suits better the specific goals of the experiment. Surgeries involving load-bearing conditions and perhaps requiring stabilization with internal or external fixation devices command a high level of surgical expertise. The maintenance of large mammals can be expensive. It is often useful to limit the number of experimental variables to reduce random effects and to ensure as much statistical significance as possible. Variables such as physical condition of the animal (nutritional status, diet, age, and sex); administration of anesthesics and/or analgesics; type of bone defect (anatomic location or use of fixation devices); and methodologies used to assess sample collection and characterization may influence the final outcome. Despite these issues, preclinical tests should be performed in large animals. Sheep or goat, for example, has a metabolism, weight, and a bone remodeling rate similar to humans.[12,18,186,189,239]

In vivo *bone regeneration studies with chitosan material*

As described above, chitosan is already used in medicine as a biomaterial for wound dressings. However, there are several reports in the literature showing the ability of chitosan to be used as material to regenerate bone (Table 2). The first report describing an attempt to regenerate bone *in vivo* using chitosan dates to 1988,[111] when Muzzarelli and colleagues implanted chitosan membranes and chitosan ascorbate gel into cranial defects in cats. Their findings suggested that chitosan seems to induce a stimulatory and/or attractive effect on stromal cells of surrounding tissues. Subsequent studies from the same authors describe the use of methylpirrolidone

TABLE 2. SURVEY OF THE *IN VIVO* STUDIES WITH CHITOSAN-BASED SCAFFOLDS PROPOSED IN THE LITERATURE FOR BONE TISSUE ENGINEERING APPLICATIONS

Scaffold structure	*Processing method*	*Cell type (source)*	*Animal model*	*References*
Methylpyrrolidinone chitosan sponges	Freeze-drying	—	Human, dental application	138
Methylpyrrolidinone chitosan sponges	Freeze-drying	—	Rabbit, tibia defect	139
Modifyed chitosan with imidazole groups sponges	Freeze-drying	—	Sheep, femur defect	140
Chitosan-HA membranes	Paste	—	Rat, implanted over calvaria	259
Chitosan-PLLA scaffolds	Freeze-drying	Rat calvaria cells	Rat, calvaria defect	260
Chitosan-gelatin-TCP scaffolds	Freeze-drying	—	Subcutaneous implantation	144
Chitosan membrane	Wet spinning	—	Dog, dental application	142
Chitosan nanofiber membrane	Electrospinning	MG63 human cell line	Rabbit, calvaria defect	116
Chitosan-alginate scaffold	Freeze-drying	MG63 human cell line	Rat, intramuscular	159
Chitosan scaffolds	Particle agregation	—	Rat, intramuscular	261
Chitosan-nanoHA	Particle agregation	Human fetal osteoblasts	Rat, calvaria defect	52
Chitosan-silk scaffolds	Freeze gelation	—	Sheep, rib defect	48
Chitosan gel	Freeze-drying-dilution in acetic acid	—	Rat, calvaria defect	262
Chitosan-PLAGA scaffolds	Particle agregation	—	Rabbit, ulna segmental defect	263
Chitosan-PBS scaffolds	Compression molding/salt leaching	Human BMSCs	Nude mice, calvaria defect	234

chitosan in defects created in the rabbit tibia[140] and in the sheep femoral head.[141] These studies confirmed previous observations[111] of the possible stimulatory and/or attractive effect of chitosan on adjacent cells. Chitosan has also been used as carrier for growth factors, such as PDGF-BB, to promote bone formation in a critical-sized calvaria defect in rats.[208,254] Osteoconductive chitosan/tricalcium phosphate (TCP) sponges were observed to promote osseous healing of rat calvarial defects versus controls (without scaffolds). The addition of PDGF-BB to a carrier further enhanced bone regeneration.[254] These authors observed that chitosan/TCP sponges without bioactive PDGF-BB resulted in more bone formation in these versus chitosan-TCP without the bioactive agents.[208] PDGF growth factor is produced by platelets, osteoblasts, and monocytes/macrophages and it is believed to have a role in the migration of MSCs to wound sites.[255] The combination of chitosan-PBS scaffolds with human BMSCs implanted into critical-sized cranial defects in nude mice resulted in enhanced integration with the surrounding tissue and significant bone formation. This was more evident for the scaffolds cultured with human cells.[232] Electrospun chitosan nanofiber membranes evidenced new bone formation at 4 weeks in rabbit cranial defects compared to the controls (empty bone defects), where only soft tissue formation was observed.[116] Chitosan combined with nanoHA, in the form of microspheres, implanted in rat calvaria defects for 12 weeks were observed to promote bone regeneration.[52] Moreover, chitosan-PLAGA microspheres conjugated in a scaffold by particle aggregation, with or without heparin and recombinant human bone morphogenetic protein 2 (rhBMP-2), promoted bone regeneration *in vivo*, with more pronounced results for the scaffolds with the incorporated growth factor.[256] A study by Ríos and coworkers[48] used a model mimicking the clinical bone free flaps, by using a cranial flap that involves the design of the desired tissue at an ectopic site in the patient's own body. This study used chambers containing silk fibroin-chitosan scaffolds implanted on top of the grafted periosteum over the *latissimus dorsi* muscle of sheep.[48] Bone grafts were used as positive controls and empty defects as negative controls. The authors found that the same amount of bone was regenerated in the defects with the tested scaffolds, as for the defects with bone grafts.[48]

Conclusions and Final Remarks

Recent developments in bone tissue engineering have been considerable, but as yet there are no bone tissue-engineered products in widespread clinical use. Both cells and biomaterial components need to be optimized to produce a functional bone tissue-engineered therapy.

New stem cell sources are being explored, such as extra-embryonic tissues, placenta, amniotic fluid, and umbilical cord. These stem cells have been shown to express pluripotent markers and low immunogenicity, evidencing a more primitive state. These cells are often discarded, which makes these sources attractive candidates for tissue engineering applications. Moreover, their low immunogenic potential could enable the use of these cells as an allogenic cell source for successful bone repair.

A new generation of biodegradable natural biomaterials is emerging, with chitosan being one of the most interesting. Chitosan has been extensively studied as a biomaterial for

bone tissue engineering scaffolds, but in practice it is still and only used as a wound dressing and hemostatic agent in medicine. Several morphologies can be successfully obtained by different processing techniques, which make this material attractive for producing scaffolds. Several studies report the biological enhancement of scaffolds with the addition of chitosan and its influence over osteogenic differentiation and bone regeneration; however, the mechanism of action remains unclear. It is worthwhile to continue to pursue research with this interesting natural polymer to clarify its function over cell performance, as well as, to improve scaffold manufacturing methodologies that could lead to its clinical use in the bone regeneration field.

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