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Review

Chitosan composites with inorganics, morphogenetic proteins and stem cells, for bone regeneration

Riccardo A.A. Muzzarelli*

Emeritus of Enzymology, University of Ancona, IT-60123 Ancona, Italy

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ABSTRACT

Resorbable composites are investigated as a means to regenerate bone lost to disease or trauma without auto- or allografts. Nano-sized hydroxyapatite, among various inorganics in composite preparations, is useful for enhancing the biochemical significance and the performances of chitosan in terms of cellular differentiation and proliferation. Composites are also envisaged to include bone morphogenetic proteins and drugs. Coatings of titanium prostheses with the aid of chitosan and hydroxyapatite permit to obtain lasting integration with living bone. Chitosan + silicate hybrids were also synthesized using γ-glycidoxypropyltrimethoxy silane whose epoxy group reacts with the amino group of chitosan. Major aspects of chemical relevance are composition, porosity, surface area and topography of the composites, along with degree of acetylation, molecular size, purity and chemical modification of chitosan.

Contents

1. Introduction

The regenerative medicine intends to restore the functions of damaged tissues and organs. Efforts are being made towards artificial tissues, including bone *in primis*, cartilage, nerve, blood vessels, and skin. The material chemistry and the biochemical technology have progressed from the use of biomaterials that may repair or replace diseased or wounded tissues to the implantable seeded supports. Scientists aim at generating or inducing the formation of a defined tissue in a certain location through selection and manipulation of cells, matrices and biological stimuli. Because these constructs mimic viable tissues, they should be functionally, structurally and mechanically comparable to the healthy tissues. The composites favor cell colonization, migration, growth and differentiation, and in certain cases guide the development of the tissue or deliver drugs and factors. The scaffold, polymers, ceramics or composites must also possess defined porosity, large surface area, adequate structural strength, and timely biodegradability. Significant results have been obtained in the development of surgical techniques for skeletal reconstruction; moreover, regenerative medicine would be an alternative

* Tel.: +39 071 36206; fax: +39 071 36206.
E-mail address: Muzzarelli.raa@gmail.com
URL: http://www.chitin.it/.

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to the conventional autogenic or allogenic bone and cartilage transplants.

Cells harvested from donor tissues, including adult stem cells, are cultured and associated with resorbable biomaterials most often of natural origin and then implanted in the desired site where the defect is regenerated consequent to optimal interactions with the host tissue. Bioactive factors can be employed to stimulate tissue growth and differentiation. In fact, exogenous cells can be treated *in vitro* with growth and differentiation factors before implantation, or, as an alternative, the factors are loaded into the matrix, to provide a suitable environment for the *host cells*. The scaffold *also* serves as a template for cellular interactions and for the formation of the extracellular matrix, acting as a structural support for the progressively regenerated tissue. A scaffold mimicking the physiological functions of the matrix is essential for cellular differentiation into their native phenotypes and for filling the tissue lesion. This explains why the scaffold should be non-immunogenic, non-toxic, biocompatible and biodegradable. The structural properties of the support affect not only cells survival, signaling, growth, propagation and reorganization, but also contribute to modulating cell shape and gene expression.

Scaffold should possess an interconnected and diffuse porosity (usually over 90%), if cell adhesion, ingrowth and reorganization are to be sustained *in vitro*, and room for neo-vascularization has to be provided *in vivo*; pore interconnections influence the diffusion of nutrients to the cells. Because excessive pore size means decreased internal surface area, a compromise is necessary: for instance, for regenerating bone tissue in vitro, some authors preferred pore size 200–400 μm, while others used scaffolds with 500 μm nominal pore size. When pore diameter is too small, pore occlusion prevents cellular penetration into the scaffold: as a consequence, pore size 75–100 μm resulted in ingrowth of non-mineralized osteoid tissue, while even smaller pores were penetrated only by fibrous tissue. Because highly porous materials have limited mechanical strength, the void volume must be tuned to allow for the accommodation of a large number of cells and the preservation of the structural strength particularly in load-bearing tissues. A proper porosity may improve mechanical interlocking between the scaffold and the surrounding host tissue, providing necessary mechanical stability at this critical interface. Scaffold surface properties such as morphology, hydrophilicity, zeta-potential and surface energy *influence* cell adhesion, migration, phenotype maintenance and intracellular signaling *in vitro*, as well as cell recruitment at the tissue / scaffold interface *in vivo*.

Table 1 collects the main characteristic properties of the natural biopolymers of interest in this area: it should be underlined that chitosan has the capacity to form complexes with both inorganic and biochemical substances. In turn, the inorganic complexes favor correct biomineralization, and chitosan-glycosaminoglycan complexes concentrate and retain growth factors.

The sudden upsurge of interest in chitosan as the main ingredient of composites and delivery vehicles intended for bone healing and regeneration, is explained by the correspondence of the unique characteristic properties of chitosan to the desiderata of research workers interested in the topics briefly recalled above. Thanks to inherent and optionally tunable cationicity, ample availability in various commercial grades, and widely recognized safety, the chitins and chitosans, together with some of their derivatives obtained by chemical or enzymatic means, are today protagonists in the scenario of wound healing (Table 2).

A valid motivation for writing today a review article on the title matter is the expectation for further important developments announced by novel chemical and pre-clinical approaches on cell differentiation agents carried by chitosans, recognition of the importance of cell receptors in investigations dealing with chitosan composites, exploration of the genetic consequences of the use of certain composites, and the growing experimental use of stem cells.

Table 1
Favorable and unfavorable properties of natural biopolymers prepared for applications in regenerative medicine (pharmaceutical and medical grades).

Chitosan = Unique cationic behavior. Hydrophilic surface promoting cell adhesion, proliferation and differentiation. High filmogenicity. Good biocompatibility and good host response. High biochemical significance in hemostasis, angiogenesis, macrophage activation, fibroblast proliferation control. Biodegradability by lysozyme and other enzymes. Bactericidal/bacteriostatic activity. Mechanical weakness. Capacity to maintain a predefined shape after cross-linking.
Silk fibroin = Slow degradability, versatility in processing, remarkable mechanical strength. Genetically tailorable composition and aminoacid sequence. Residual sericin may cause biocompatibility problems.
Collagen = Low antigenicity and good cell-binding properties. Collagen type I (the most abundant extracellular matrix protein) supports cell adhesion and proliferation; integrin-mediated adhesion to collagen type I enhances osteogenic differentiation of human bone marrow mesenchymal stem cells. Low biomechanical stiffness and rapid biodegradation.
Hyaluronan = Absence of immunogenic properties. Easy chain size manipulation. Interactions with cell-surface receptors. Production through large-scale microbial fermentation. Its anionic surface does not promote cell attachment and tissue formation. Very soluble in water. Quick degradation by lysozyme and other enzymes
Alginate = Cross-linking under very mild conditions. Suitable for gel injection. Mechanical weakness. Difficulties in handling and sterilization. Variety of structures
Starch = Inexpensive. *In vivo* degradation has not been fully assessed yet
Bacterial cellulose = High purity, nanofibrous structure, high tensile strength and good biocompatibility. Small pore size. Unclear *in vivo* behavior
Dextran = Susceptible to chemical modification, suitable for designing of scaffolds with specific sites for cell recognition. Shortcomings typical of hydrogels. Needs modification to enhance cell adhesion. Scarce characterization

Table 2
Selected books and review articles containing basic information relevant to chitosans.

On the biomedical applications: Cui, Zhang, Wang, & Gao, 2008; Dorozhkin & Epple, 2002; Jayakumar et al., 2010; Klokkevold, Vandemark, Kennedy, & Bernard, 1996; Lee & Mooney, 2001; Mourino & Boccaccini, 2010; Muzzarelli, 2009a; Muzzarelli, 2009b; Muzzarelli et al., 1998; Swetha et al., 2010; Puppi, Chiellini, Piras, & Chiellini, 2010; Suphasiriroj, Yotnuengnit, Surarit, & Pichyangkura, 2009; Xu, Quinn, Takagi, & Chow, 2002
On the chemical and enzymatic properties: Castagnino et al., 2008; Kurita, 2006; Jollès & Muzzarelli, 1999; Muzzarelli, 1977; Muzzarelli, 2009a; Muzzarelli, 2009b; Muzzarelli & Muzzarelli, 2005a; Muzzarelli & Muzzarelli, 2005b; Muzzarelli & Muzzarelli, 2009; Mourya & Inamdar, 2008; Sahoo, Sahoo, Mohanty, Sasmal, & Nayak, 2009; Varlamov, Bykova, Vikhoreva, Lopatin, & Nemtsev, 2003
On the pharmaceutical, cosmetic and dietary properties: Calandrelli et al., 2010; Degim, 2008; Keong & Halim, 2009; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli, 2010; Muzzarelli & Muzzarelli, 2006; Sogias, Williams, & Khutoryanskiy, 2008; Xu, Chao, & Wan, 2009
On the chemical structure: Muzzarelli & Muzzarelli, 2005a; Muzzarelli & Muzzarelli, 2005b; Rinaudo, 2006a; Rinaudo, 2006b; Sikorski, Hori, & Wada, 2009

In particular, the scope of the present review article is to throw light on the capacity of chitosan to form composites, among which the reactivity with polyanionic species and consequent formation of polyelectrolyte complexes; heparin, hyaluronan, alginate and poly(L-lactic acid) are important for various biochemical/technical reasons, but anionic species on the cellular surfaces, most significant for recognition, mediate stimulation by chitosans. Thus, chitosans are simultaneously effective in promoting mineralization and cell activation.

1.1. Early studies in dentistry and experimental surgery

The reconstruction of the periodontal tissue with chitosan was a prelude to the discovery of the osteoinductive properties

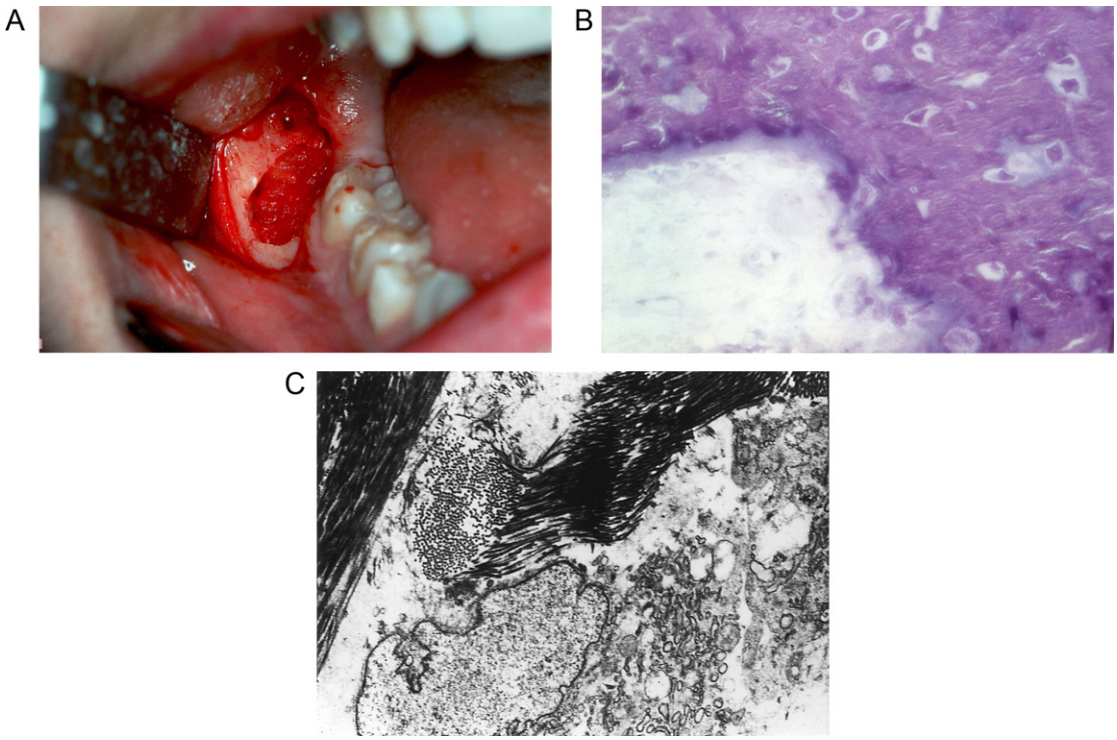

Fig. 1. A. Wisdom tooth avulsion in a patient suffering from dysodontiasis involving the third inferior molar. Gel formation by methylpyrrolidinone chitosan takes place immediately after insertion: the red coloured gel fills the cavity from which the tooth has been removed after resection of the alveolar crest. The wound is ready for suture (Original photograph by the author, 1990). B. After chitosan gel resorption, newly formed osseous trabecular structures characterized by the presence of abundant osteocytes are revealed at the morphological analysis (Original photograph by the author, 1990). C. Newly formed trabecula with bundles of collagen fibres and a mesenchymal–osteoblastic cell element in the healing avulsion site (Original photograph by the author, 1990).

of chitosan. In surgical wounds from wisdom tooth avulsions, bone regeneration was promoted with freeze-dried methylpyrrolidinone chitosan. The initial steps of the bone regeneration are illustrated in Fig. 1A–C; mineralization followed shortly. The polysaccharide gel was depolymerized by lysozyme *in vivo* and was no longer detected 6 months after surgery. Said chitosan was found to be useful in apicectomy as well, based on radiographic evidence without adverse effects over a 3 year observation period.

The existence of osteoprogenitor cells in a wound site with healthy tissues, such as the avulsion site, offers the possibility of regenerating the periodontal, peri-implant, and alveolar ridge bone tissues simply with the aid of chemical mediators from chitosan: bone-forming colonies almost doubled in the presence of chitosan. In fact, chitosan stimulates the differentiation of osteoprogenitor cells thereby facilitating the formation of bone (Muzzarelli et al., 1989; Muzzarelli, Biagini, et al., 1993; Muzzarelli, Biagini, Mattioli-Belmonte, et al., 1993). Further studies extended the evaluation of chitosan to periodontitis, a chronic infection in the supportive tissue of the tooth which eventually leads to tooth loss. In 20 chronic periodontitis patients, radiographic data revealed that chitosan gel alone or its combination with demineralized bone matrix/collagen membrane is suitable for periodontal regeneration (Boynuegri et al., 2009; Tigli et al., 2009).

In fact, a bioactive three-dimensional scaffold for the promotion of cellular proliferation and differentiation is quite useful in periodontal tissue engineering. Freeze-dried porous β-tricalcium phosphate + chitosan composites with 120 μm pore size and 91.07% porosity showed higher proliferation rate than the pure chitosan, and up-regulated the gene expression of bone sialoprotein and cementum attachment protein. *In vivo*, human periodontal ligament cells in the composite not only proliferated but also recruited vascular tissue ingrowth. The expression of alkaline phosphatase and osteopontin was up-regulated in the composite (Liao et al., 2010).

With the intention of improving bone tissue reconstitution with chitosan + calcium phosphate composites, experimental surgery on rabbit and sheep was performed. Histological evidence showed the presence of an osteogenic reaction moving from the rim of the surgical lesion toward the center. In controls, dense fibrous tissue deprived of the characteristic histoarchitecture of bone was observed. In an osteoporotic experimental model, the bone morphogenetic protein linked to the chitosan improved the bone tissue regeneration in surgical bone defects (Muzzarelli et al., 1997).

Dicarboxymethyl chitosan applied to surgical femoral defects for 21 days produced a good histological order in the newly formed bone tissue. The effects of dicarboxymethyl chitosan on the precipitation of a number of insoluble salts, including calcium phosphate, were studied: the chelating ability of the modified chitosan interfered with the chemical behavior of magnesium and calcium salts. Dicarboxymethyl chitosan mixed with disodium hydrogen phosphate in suitable ratios with calcium acetate yielded solutions from which an amorphous material (ca. 50% inorganic) was isolated for treatment of bone lesions in dentistry and experimental surgery. Bone regeneration was promoted in sheep, leading to healing of otherwise non-healing surgical defects. The dicarboxymethyl chitosan + calcium phosphate chelate favored osteogenesis and promoted bone mineralization. The *in situ* precipitation route toward obtaining composites of polymer and calcium phosphate proved to be a viable procedure for the synthesis of bone substitutes, because it is similar to the strategy employed in naturally occurring biocomposites (Mattioli-Belmonte et al., 1999; Muzzarelli & Muzzarelli, 2002).

Likewise, 6-oxychitin sodium salt, obtained via regiospecific oxidation at C6 as partially depolymerized (1–4)β-2-acetamido-

2-deoxy glucuronic acid sodium salt, when applied to surgical femoral defects, generated a novel histoarchitectural order in the newly formed bone within 3 weeks. The spongious trabecular architecture was restored; moreover the composite of 6-oxychitin with osteoblasts led to outstanding performances in bone recovery (Muzzarelli et al., 2001).

Because it is well tolerated by the synovia, chitosan was used to assist the spontaneous but difficult repair of the meniscus, where in fact it favored and stimulated repair processes that do not take place spontaneously. Its initial angiogenetic action appeared to be effective enough to provide the meniscus with the necessary tissue components and humoral factors. As a continuation of these early observations, surgical bone defects in sheep and rabbit models were treated with freeze-dried methylpyrrolidinone chitosan. Bone osteoid formation was followed by mineralization; osteoinduction was also observed in rabbit endochondral bones (Borah, Scott, & Wortham, 1992; Mattioli-Belmonte et al., 1995; Muzzarelli, Bicchiega, Biagini, Pugnaloni, & Rizzoli, 1992; Muzzarelli, Biagini, et al., 1993; Muzzarelli, Biagini, Mattioli-Belmonte, et al., 1993).

The early original approaches evolved into elegant and refined preclinical studies: for example, important advances have been made in elucidating the effects of the combination of platelet-rich plasma and chitosan on bone regeneration in experimental rabbit cranial defects. The latter were filled with thick trabecular new bone, and more conspicuous bone formation was observed in the platelet-rich plasma group when compared with control groups. The work by Oktay et al. (2010) is a continuation of the one by Chang, Kuo, and Lan (2009) who proposed platelet-rich plasma as a beneficial osteogenic substance with applications in periodontal diseases. With stereolithography, photo-curable chitosans were shaped into scaffolds with desired topography, thanks to their good solubility in organic solvents. The content of photocurable groups in the chitosan could be controlled by adjusting the feeding ratio of the raw components. Fibroblast cultures indicated that these photo-cured chitosans are cyto-compatible. Scaffolds with interconnected pores, fabricated using salt leaching and photo-curing and seeded with fetal bovine osteoblasts, were then implanted subcutaneously in the backs of athymic *rat: histological observation at 6* weeks showed good biocompatibility and osteoconductivity (Qiu, Zhang, Kang, An, & Wen, 2009).

2. Bone substitutes and injectable cements

Degradable matrices optimize the osteoconductive behavior of hydroxyapatite, allowing bone ingrowth into the implant to occur as the matrix is progressively resorbed with time; the degradable matrices prevent loss of hydroxyapatite from the implant. The chitosan bonded hydroxyapatite bone-filling paste was initially made as follows: chitosan (0.5 g) was dissolved in malic acid (0.5 g) solution made with saline, and a chitosan film was formed by mixing this solution with hydroxyapatite powder (2 g), followed by neutralization with 5% sodium polyphosphate. The tensile strength and elongation were optimized to help cells and blood vessels to penetrate this material (Ito & Hidaka, 1997; Ito, 1991; Maruyama & Ito, 1996). Similarly, a composite of hydroxyapatite and a network formed via cross-linking of chitosan and gelatin was made with glutaraldehyde by Yin et al. (2000). The formation of the chitosan/gelatin network was not retarded by the hydroxyapatite.

The calcium phosphate cements are suitable for bone repair and regeneration because after implantation in bone defects they are rapidly integrated into the bone structure, after which they are transformed into new bone thanks to the activity of osteoclasts and osteoblasts. These cements have the advantage that they can be moulded during the operation and are injectable, i.e., they fill the bone defect and permit subsequent good osteointegration; the amorphous state of the inorganic compound is important in this respect because it permits immediate elaboration into the physiological biomineral crystalline form and shape. To make the cement injectable, several additives can be used; however, the properties of the cement should be preserved, namely: setting times suited to a convenient delay with surgical intervention, limited disintegration in aqueous medium, and sufficient mechanical resistance. Chitosans, lactic acid, glycerol and glycerophosphate are adjuvants in terms of injectability, disintegration, setting time, and toughness (Leroux, Hatim, Freche, & Lacout, 1999). These results have been reviewed recently by Low et al. (2010) and by Martins, Alves, Kasper, Mikos, & Reis (2010). According to Danilchenko et al. (2010) the formation of the calcium phosphate mineral in chitosan solution is substantially modulated by the chemical interaction of the components; apparently, a part of calcium is captured by chitosan and does not participate in the formation of the main mineral phase. The apatite in the composite is calcium-deficient, carbonate-substituted and is composed of dispersed nano-sized crystallites, i.e. has properties that closely resemble those of bone mineral.

The preparation of bone fillers evolved over the last decade (Geffre, Ochoa, Margolis, & Szivek, 2010): a recently proposed injectable bone substitute consisting of citric acid, chitosan solution as the liquid phase and tetracalcium phosphate, dicalcium phosphate anhydrous and calcium sulfate hemihydrate powders as the solid phase was prepared. Four groups containing up to 30% of $CaSO_4 \cdot 0.5H_2O$ showed that the setting time for all compositions was in the range of 25–45 min, and that the injectability was improved by the addition of this salt. The XRD peak intensity of hydroxyapatite increased with time and quantity of $CaSO_4$: the enhancement of crystallinity was further confirmed by SEM. The maximum compressive strength obtained for the bone substitute was with 20% $CaSO_4$ after 28-day incubation in 100% humidity at 37 °C. Chiang, Ho, Chen, Lai, & Ding (2010) developed a cement consisting of a chitosan oligosaccharide solution in a liquid phase and gelatin containing solid calcium phosphate powder. Chitosan oligosaccharides enhanced the cement biocompatibility as an approximate twofold increase in cell proliferation for 10% chitosan-containing cements was observed as compared with the controls. The combination of gelatin and chitosan oligosaccharides provided benefits due to synergistic effects in terms of anti-washout properties and biological activity (Song, Rahman, & Lee, 2009).

3. Composites containing hydroxyapatite

Biodegradable scaffolds are being investigated as a way to regenerate bone without the need for auto- or allografts. Along this line, it was found that facile plasma-induced grafting of phosphonic groups onto chitosan leads to remarkable cellular response and significantly improves adhesion, proliferation and viability of osteoblasts (Lopez-Perez, da Silva, Serra, Pashkuleva, & Reis, 2010; Wilson & Hull, 2008). Composites made of phosphorylated chitosan + chitosan + hydroxyapatite (weight ratios 10/30/60) had bending strength ca. 35 MPa, i.e. 1.6 times higher than the cancellous bone (Li, Wang, Ma, & Huang, 2009).

Hydroxyapatite powder with size distribution 3.0 ± 0.1 μm, similar to the innate hydroxyapatite in human bone (57.0 and 41.4 of CaO and P_2O_5, respectively; Ca/P molar ratio 1.67) was used to prepare films after suspension in chitosan (400 kDa) solutions: the film appeared white and flexible with transmittance <20% in the visible range, and monodisperse under the SEM. Monodispersity is an indication of the homogeneity and uniformity of the film, and absence of phase separation. Chitosan influence on osteoclast differentiation, which plays also a central role in bone remodeling, was recently described. The differentiation and activity of human pre-osteoclastic cells on calcium phosphate cement containing 2%

chitosan (deacetylation degree 0.83) (Cementek®/chitosan) was compared to the Cementek® alone. Incorporation of chitosan to Cementek® did not affect the proliferation and adhesion of preosteoclasts but prevented the osteoclastic resorption of the composite biomaterial, and the dramatic inhibition of the tartrate-resistant acid phosphatase enzymatic activity of the cells attached on the composite, compared to the cells attached to the cement alone. This property of chitosan may positively influence bone formation and bone remodeling *in vivo* (Rochet et al., 2009). Multi-layered films of hydroxyapatite and chitosan were prepared by Sun, Lim, Ryu, Lee, & Lee, 2010.

Needle-like hydroxyapatite nanocrystals with low degree of crystallinity were uniformly embedded in the chitosan matrix, when the composite was prepared in the form of membrane by coprecipitation and freeze-drying. The tensile strength of the membrane was inversely dependent on the hydroxyapatite content, while the elastic modulus was at a maximum when hydroxyapatite was present at 20% by weight. The highest alkaline phosphatase level was achieved in cell cultures when hydroxyapatite was 30% in the composites (Teng et al., 2009).

Hydroxyapatite forms composites with chitosan derivatives as well. The pores of the composite made of crystalline hydroxyapatite and carboxymethyl chitosan were regular, interconnected, with size in the range of 20–500 μm; diffraction peaks characteristic of apatite, and typical bands from carboxymethyl chitosan showed that coprecipitation of both ingredients was effective. In fact, the composite scaffolds exhibited 58.9% ± 6% porosity, and consisted of 24% hydroxyapatite and 76% carboxymethyl chitosan: it was degradable and bioactive (Oliveira et al., 2009).

The three-component scaffold manufactured by Li, Kommareddy, et al., 2010, consisting of hydroxyapatite microspheres, poly-L-lactic acid and chitosan had a macroporosity of more than 50%; compressive strength and elastic modulus (0.42 and 1.46 MPa, respectively) were much higher than those of chitosan/hydroxyapatite composites. These scaffolds showed excellent biocompatibility and supported 3D growth of pre-osteoblastic cells, that formed a network on the hydroxyapatite microspheres and proliferated not only in the macropore channels but also in the micropores. The proliferating cells formed an extracellular matrix network and also differentiated into mature osteoblasts, as indicated by alkaline phosphatase activity (Venugopal et al., 2010).

3.1. Advantages of nano-hydroxyapatite

The geometry, size and crystallinity degree of the particles, as well as the surface topography are able to influence cellular behavior: in fact the cell attachment depends on the degree of roughness of the material surface. As the roughness of the surface increases, the number of adherent cells increases. Recent results indicated that the cytocompatibility is promoted when the roughness is about 36 and 54 nm (arithmetic mean deviation of the surface) and 115 and 227 nm (max. mean peak to valley height of the surface) the lower values being for the nano form and the higher values for the micro form, respectively. While a too smooth surface does not adsorb much protein, a too rough surface with deeper grooves influences the migration behavior of cell. As the cells occupied the grooves rather than the ridges and adhered to lower parts of the patterned surface, relative roughness of micro-hydroxyapatite + chitosan + gelatin surface made the migration difficult (Li, Zhu, et al., 2009).

The uniform dispersion of the nanoparticles in chitosan solutions was recognized as an important motif for innovative preparation of chitosan composites: in fact, nano-hydroxyapatite + chitosan membranes were found suitable for guided bone regeneration. The nano-hydroxyapatite used by Chen, Wang, & Lin (2002) was ca. 20–30 nm in width and 50–60 nm in length, with specific surface area of 73 m^2/g; aggregation was avoided with the use of n-butanol, and the nano-hydroxyapatite was prepared according to the chemical equation: $Ca(NO_3)_2{\cdot}4H_2O + (NH_4)_3PO_4{\cdot}3H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2$. While the pH value was kept above 10 with ammonium hydroxide, the precipitated apatite was treated at 100 °C under normal atmospheric pressure for 3 h. After treatment, the apatite turned to needle-like nano-crystals. After washing to neutrality, the slurry containing 10 wt.% nano-hydroxyapatite and 90% deionized water was chosen to make the composite membrane with chitosan (400 kDa; deacetylation 0.95). The tensile strength [MPa] and elongation rate [%] of wet membranes with hydroxyapatite to chitosan ratio of 4:6, at 30, 60 and 90 °C were respectively 9.43 and 56; 10.56 and 65; 4.96 and 20. The surface roughness and micropores of the composite membranes increase with the rise of nano-hydroxyapatite content, suitable for adhesion and growth of cells. Chemical bonds were present between Ca ions and hydroxyl groups of hydroxyapatite and chitosan. The hydroxyapatite content influenced the proliferation of the cells: the composite membrane had no negative effect on the bone marrow stromal cell morphology, viability and proliferation, and exhibited good biocompatibility (Cheng et al., 2009).

To produce a biodegradable porous composite, nano-hdroxyapatite (73–136 nm) was crystallized *in situ* on the organic polyelectrolyte complex matrix through a biomimetic method. Significant enhancement of the pre-osteoblast attachment, proliferation, and widespread morphology in relation to pure chitosan was observed for biodegradable chitosan-nanohydroxyapatite composites fabricated via lyophilization to have highly porous structure and pore size of ca. 50–125 μm. Considering that the surface characters of the composites influence their biological properties, nano-hydroxyapatite + chitosan + gelatin films were prepared via biomineralization of chitosan + gelatin network films in $Ca(NO_3)_2 + Na_3PO_4$ tris buffer solution. The micro-hydroxyapatite + chitosan + gelatin films were formed via immersing the chitosan + gelatin network films into the 5 μm hydroxyapatite suspensions. The ion/polar interactions were the main driving forces for nano-hydroxyapatite composite formation via biomineralization. The hydrogen bonds between the carboxyl, hydroxyl and amino groups of the chitosan + gelatin films and hydroxyl groups of hydroxyapatite played an important role in the formation process of the composite. Said composites had excellent biocompatibility, and the nano form presented higher osteogenic differentiation activity than the micro form, as already mentioned (Chen, Wang, & Chen, 2009; Li, Zhu, et al., 2009; Thein-Han & Misra, 2009a; Thein-Han & Misra, 2009b).

Likewise, the same *in situ* synthesis approach was adopted to prepare nanocomposites of collagen + chitosan. Structural investigations of the pure mixture validated the influence of chitosan on collagen assembly, but the molecular interactions between chitosan and collagen were partially depressed during the intervention of *in situ* synthesis of hydroxyapatite. A series of collagen + chitosan + hydroxyapatite nanocomposites with varying hydroxyapatite content were thereby prepared by a sequential method, involving *in situ* synthesis, gelling at 25 °C, washing the resultant elastic gel, and consolidating via dehydration. A well integrated microstructure of organic fibres (ca. 90 nm in size) and dense matrix including inorganic aggregates was formed in these nanocomposites. Rat osteoblasts attached and proliferated on the surface of both nanocomposite and collagen+chitosan mixture (Wang et al., 2009).

High- and medium-MW chitosan (degree of deacetylation 0.83) composites lyophilized with 0.5, 1 and 2% of nano-hydroxyapatite exhibited highly porous structure and pore size of ca. 50–120 μm.

The biological response of pre-osteoblasts on nanocomposite scaffolds was superior in terms of improved cell attachment, higher proliferation, and well-spread morphology compared to chitosan. In composites, cell proliferation was about 1.5 times greater than pure chitosan after 7 days of culture and beyond, as documented by an impressive series of microphotographs illustrating the morphology of the seeded pre-osteoblasts. It was suggested that hydroxyapatite nanoparticles on the scaffold surface influence the morphology of attached cells, based on the adsorption of integrins on hydroxyapatite. In general, cells attach to extracellular matrix proteins such as fibronectin and vitronectin using integrin molecules. Hydroxyapatite adsorbs fibronectin and vitronectin from serum thus enhancing protein adsorption, with subsequent binding of integrins and osteoblast precursors (Thein-Han & Misra, 2009a; Thein-Han & Misra, 2009b).

Human osteosarcoma cells (MG-63) were seeded in composites prepared by blending chitosan and gelatin with nano-hydroxyapatite. The composites were highly porous with a pore size of 150–300 μm, had good swelling character, low degradation rate and increased mineralization in simulated body fluid. The biological response of MG-63 cells in nanocomposites was superior in terms of improved cell attachment, higher proliferation, and spreading compared to chitosan–gelatin scaffold (Peter et al., 2010). A composite material consisting of nano-hydroxyapatite and collagen was produced as a spongy construct by biomimetic precipitation: the nano size was confirmed to be important for achieving the homogeneous resorption of the material by osteoclasts (Nitzsche et al., 2010). A ZnO containing nano-hydroxyapatite/chitosan cement showed excellent injectability during the first 4 min. Four weeks after injection in the rabbit tibia, the inflammation began to disappear and the cement, bound to the surrounding host bone, induced new bone formation (Li, Li, Yi, Lan, & Jansen, 2010).

Chitosan + 2-glycerophosphate salt formulations were combined with bioactive glass nanoparticles in order to prepare novel injectable thermo-responsive hydrogels for orthopaedic applications. The rheological properties of the developed organic-inorganic *in situ* thermosetting systems were adequate for intra-corporal injection. *In vitro* bioactivity tests, using incubation protocols in simulated body fluid, revealed bone-like apatite formation in the hydrogel formulations containing nanoparticles. The density of the apatite formed increased linearly with bioactive glass content and soaking time, indicating that the stimuli-responsive hydrogels were suitable as temporary injectable scaffolds in bone tissue engineering. Nano-hydroxyapatite + chitosan bone cement was selected to generate a drug delivery system for the treatment of bone defects. The setting time of the cement ranged from 17.03 ± 0.50 min to 28.47 ± 0.96 min and the compressive strength changed from 184.00 ± 7.94 to 120.33 ± 9.02 MPa with the increase of berberine, an antibacterial alkaloid, in the composite. The *in vitro* release of berberine could last more than 4 weeks (Couto, Hong, & Mano, 2009Couto, Hong, & Mano, 2009). Two short review articles have recently addressed the formulation of biocomposites containing natural polymers and hydroxyapatite (Swetha et al., 2010), and the use of carbon nanotubes (Sahithi, Swetha, Ramasamya, Sriniyasan, & Selyamurugan, 2010). Graphene + chitosan acetate films were tested by the nanoindentation method: for 0.1–0.3 wt% graphene in chitosan, the value of the elastic modulus of chitosan doubled. The L929 cells adhered to and developed on the graphene + chitosan acetate films as well as on pure chitosan film, indicating that the composite had good biocompatibility. No time-consuming purification is necessary because metallic impurities are absent in graphene, as a point of difference from carbon nanotubes (Fan et al., 2010).

4. Functionalization of titanium surface

Orthopedic implant failure has been attributed mainly to loosening of the implant from host bone, which may be due to poor bonding of the implant material to bone tissue, as well as to bacterial infection. One promising strategy to enhance tissue integration is to develop a selective biointeractive surface that simultaneously enhances bone cell function while decreasing bacterial adhesion. *In vitro*, the surface of titanium alloy was functionalized by first covalently grafting carboxymethyl chitosan, followed by the conjugation of the latter with the bone morphogenetic protein-2. The adhesion of *Staphylococcus aureus* and *S. epidermidis* on the carboxymethyl chitosan + bone morphogenetic protein-2 surface was significantly reduced compared to that on the pristine supports. In addition, the carboxymethyl chitosan + bone morphogenetic protein-2 modified supports significantly promoted attachment, alkaline phosphatase activity, and calcium mineral deposition of both osteoblast and human bone marrow-derived mesenchymal stem cells. The achievement of the dual functions of bacterial adhesion reduction and cell function promotion by the carboxymethyl chitosan + bone morphogenetic protein-2 modified titanium surfaces illustrates the good potential of such surfaces for enhancement of tissue integration and implant duration (Shi et al., 2009; Shi, Neoh, Kang, Poh, & Wang, 2009).

Chitosan was used to increase the biocompatibility of electrolytically deposited apatite coatings on titanium alloys: that coating exhibited an improved bone marrow stromal cell attachment (Pang & Zhitomirsky, 2007). Similar data were obtained for chitosan coupled with surface-immobilized cell-adhesive arginine–glycine–aspartic acid peptide, and for hyaluronan (Chua, Neoh, Kang, & Wang, 2008). Likewise, titanium was coated with three chitosans of different degree of deacetylation and from different manufacturers via silane + glutaraldehyde. Coating bond strength was in the range 2.2–3.8 MPa regardless of degree of deacetylation. The coatings did not appreciably dissolve over 5 weeks even in the presence of lysozyme, and were judged to be osteocompatible *in vitro*. Titanium coatings made of chitosan cross-linked with silane + glutaraldehyde showed increased osteoblast attachment and proliferation: the bond strength of said coating was in the range of 1.5–1.8 MPa, osseo-integration of Ti devices was promoted and its full resorption occurred in 8 weeks (Bumgardner, Wiser, Elder et al., 2003a; Bumgardner, Wiser, Gererd, et al., 2003a). A chitosan + nanocrystalline calcium phosphate composite and a plain chitosan scaffold were prepared from microspheres of 500 to 900 μm in diameter: both had porosity of 33–35% and pore sizes between 100 and 800 μm. However, composites were rougher and, as a result, had 20-fold larger specific surface area than chitosan scaffolds (Chesnutt et al., 2009). The compressive modulus of hydrated composites was significantly higher than for chitosan scaffolds (9.29 ± 0.8 vs. 3.26 ± 2.5 MPa), and composites were tougher and more flexible than what has been reported for other chitosan + calcium phosphate composites. They contained hydroxyapatite with crystallinity degree $16.7\% \pm 6.8\%$ and crystallite size 128 ± 55 nm. Fibronectin adsorption on composites was increased, and cell attachment was higher after 30 min, although attachment rates were similar after 1 h. Osteoblast proliferation increased after 1 week of culture. The composites have mechanical properties and porosity sufficient to support ingrowth of new bone tissue: cell attachment and proliferation data indicate that composites are suitable for bone regeneration (Greene, Bumgardner, Yang, Moseley, & Haggard, 2008; Wang, DeBoer, & DeGroot, 2008; Yuan, Chesnutt, Wright, Haggard, & Bumgardner, 2008).

A bioactive porous apatite + wollastonite + chitosan composite coating for titanium implants was prepared by electrophoretic deposition. The crystalline phase of apatite wollastonite composite coating was 65%; the porosity had interconnections with

good homogeneity between the phases. The presence of chitosan increased the adhesive strength of the composite coating. The Young's modulus of the coating was 9.23 GPa. During apatite growth in the presence of chitosan, homogenous nucleation was the primary factor for sheet-like evolution of the apatite layer; as a consequence, the incorporation of chitosan with apatite + wollastonite in composite coating could provide excellent *in vitro* bioactivity with enhanced mechanical properties (Sharma, Soni, & Bellare, 2009).

Circulating progenitor bone cells can home to a bone implant, differentiate, and eventually osteointegrate with the prosthesis, thus helping reduce the risk of implant failure. Immobilized bone morphogenetic protein-2 on chitosan-grafted titanium support enhanced bone marrow-derived mesenchymal cell adhesion onto the support surface and further induced their differentiation into osteoblasts. The chitosan + Ti support was able to release slowly the adsorbed bone morphogenetic protein-2. Based on the fact that alizarin red staining revealed the presence of calcium deposits in the differentiated cells, the chitosan + Ti + bone morphogenetic protein-2 supports exerted an osteoconductive effect (Lim, Wang, Shi, Poh, & Neoh, 2009; Zou et al., 2009).

5. Guided bone regeneration

Guided bone regeneration reconstructs new tissue by using a barrier membrane to guard the defected area from invasion of other tissues, especially fibrous connective tissues. This relatively simple treatment was proposed for periodontal therapy where membranes prevent apical migration of gingival epithelial cells into the bone defect and promote the growth of progenitor bone and periodontal ligament cells. Membranes for this purpose include Gore-Tex™, collagen membrane; Bio-Guide™, Vicryl Periodontal Mesh, polylactic acid sheet; and Guidor. General requirements for the barrier membranes are suitable mechanical strength, mechanical stability, optimal porosity and biodegradability. A porous structure both at the surface and in the sub-layer of the membranes is essential for cellular adaptation and sufficient nutrient permeation. Non-biodegradable synthetic membranes, such as Gore-Tex™, require a secondary surgical procedure for retrieval and this remains a significant drawback. In order to avoid the second-stage operation of removing the non-absorbable membrane and to guarantee the continuous healing of tissue, the membrane should be completely resorbable after it has performed its function. In fact, biodegradable membranes, such as synthetic polyesters and collagen, do not require secondary surgery for membrane removal. However, the degradation products of the synthetic polymers reduce the local pH, accelerate the polymer degradation rate and induce an inflammatory response. On the other hand, collagen is potentially immunogenic and can be expensive, and there can be great variations between the collagen batches.

In addition to the studies on guided bone regeneration by Teng et al. (2009) and by Cheng et al. (2009) mentioned above, most significant studies on chitosan + polycation membranes for guided bone regeneration were made by Zheng, Wei, Wang, Gong, & Zhang (2009) who blended chitosan with poly(L-lysine), poly(ethyleneimine), or poly(L-ornithine). Osteoblast-like cells on chitosan + poly(L-lysine) presented well developed cytoskeletal organization and higher adhesion, proliferation, and differentiation than on the other two composite membranes or plain chitosan, and exhibited higher phosphorylation levels of kinases; moreover, they achieved enhanced mRNA expression of fibronectin, integrin-α5, and integrin-β1, thus qualifying for use in guided bone regeneration. Because the surface of hydroxyapatite is also susceptible to osteoblast attachment, the composites of hydroxyapatite and chitosan promote osteoblastic adhesion, migration, differentiation, and proliferation (Lee, Kim, et al., 2009). On this basis, a chitosan + hydroxyapatite + fibroin composite membrane was proposed for applications in guided bone regeneration. Likewise, chitosan + silica membranes were fabricated using a sol-gel process: osteoblasts were observed to adhere well and grow better on said membrane than on plain chitosan membrane (Lee, Shin, Kim, et al., 2009). In agreement with analogous findings, the alkaline phosphatase activity of the cells was also much higher on the hybrid than on the chitosan membrane. The in vivo study of the chitosan + silica membrane in a rat calvarial model demonstrated enhanced bone regeneration, and the histomorphometric analysis performed 3 weeks after implantation revealed the complete closure of the defect (Dupoirieux, Pourquier, Picot, & Neves, 2001; Kostopoulos & Karring, 1994; Piattelli, Scarano, Russo, & Matarasso, 1996).

Of course, suitable mechanical properties are essential for satisfactory applications and should accompany biodegradability: plain chitosan films failed in this respect because of poor mechanical resistance at the time of application. The preparation of chitosan-based composite films has to confer improvements of the mechanical properties: this was also the scope of the studies on chitosan + silicate hybrid membranes mentioned below.

6. Composites containing silicate or calcium sulfate

Several multi-phase materials based on silica are intended for bone regeneration. The morphology of non-woven silica gel fabric prepared by electrospinning is suitable for the differentiation of pre-osteoblastic cells and for osteoconductivity (Kang, Kim, Seol, & Rhee, 2009). Nano-silica fused whiskers combined with calcium phosphate cements act as fillers in a composite, the role of nano-silica being to strengthen the phosphate-based composite: the mechanical properties of the phosphate + silica + whisker composites nearly matched those of cortical and trabecular bone (Xua, Smith, & Simon, 2004). The main drawback of the biocomposites of hydroxyapatite + tricalcium phosphate is a substantial decrease in the activity of calcium phosphate in physiological media. On the other hand, the advantages of such implants are their mechanical strength, comparable to that of bone, and their suitability for the production of structural implants.

In a typical approach, bone like mineral layers were deposited on the exterior surface of chitosan microparticles fabricated by double emulsification in the spherical shape and the size of 30–60 μm. The microparticles were then placed in concentrated simulated body fluid and allowed to undergo biomineralization to form a superficial bone-like mineral layer at 37 °C over a 24 h period, to be used to target drugs and to assist bone growth. As an alternative, chitosan powder (medium molecular weight, and degree of deacetylation 0.85), and tetramethylorthosilane were used to manufacture chitosan + silica membranes in which nano-silica was dispersed. The hybrid membranes showed superior mechanical properties to chitosan in the wet state and the rapid induction of calcium phosphate minerals in simulated body fluid, reflecting their excellent *in vitro* bioactivity (Jayasuriya & Kibbe, 2010).

Silica xerogel-chitosan nano-hybrids are also suitable as drug eluting bone replacement. As the content of chitosan was increased, the strength, strain to failure, and work of fracture of the silica xerogel-chitosan hybrids were significantly enhanced, while the elastic modulus was decreased. These changes in the mechanical properties were mainly attributed to the mitigation of the brittleness of the silica xerogel thanks to the flexible chitosan phase. The hybrids with at least 30% chitosan released the vancomycin for a protracted period of time in a controlled manner (Lee et al., 2010).

Chitosan + silicate hybrids were also synthesized with γ-glycidoxypropyltrimethoxy silane whose epoxy group react with

the amino groups of chitosan. The cross-linking density was around 80% regardless of the amount of silane. The hydrophilicity of the hybrids increased except when the content exceeded the molar ratio of 1.5. The values of the mechanical parameters indicated that significant stiffening of the hybrids was obtained upon addition of the silane while full flexibility was retained; the Young's modulus increased with higher quantities of the silane. The adhesion and proliferation of the MG63 osteoblast cells cultured on the hybrid surface were improved compared to those on the plain chitosan membrane, regardless of the silane concentration. Moreover, human bone marrow osteoblast cells proliferated on the chitosan hybrid surface and formed a fibrillar extracellular matrix. The same hydrogel derived from chitosan and said silane was characterized for the purpose of developing an injectable system for the application of Bonelike® using a resorbable vehicle usable in minimal invasive surgery. The Bonelike® graft is a bone substitute that mimics the inorganic composition of bone; this biomaterial was developed and characterized over the last decade. The mixture derived from chitosan and said silane existed in sol state at room temperature and formed a hydrogel at 37 °C, whose degradation was controlled by the concentrations of both ingredients. The pH changes caused by the degradation of this hydrogel were small, so it did not cause any deleterious effect *in vivo*. When cultured with bone marrow cells, the hybrids showed abundant cell growth and matrix mineralization in the presence as well as in the absence of dexamethasone: this is a relevant observation because this compound is frequently added to cell cultures to improve the proliferation and/or differentiation of osteoblastic cells in a number of cell systems (Maniatopoulos, Sodek, & Melcher, 1988; Shirosaki, Botelho, Lopes, & Santos, 2009a; Shirosaki, Tsuru, et al., 2009). The osteoblastic activity was enhanced and bone-like apatite formation on freshly lyophilized chitosan scaffolds was achieved upon immersing the composites into a concentrated simulated body fluid (10 x SBF-like solution) for various hours (Aday & Gumusderelioglu, 2010).

The hemihydrate form of calcium sulfate, $CaSO_4 \cdot 1/2\ H_2O$, better known as plaster of Paris, prevents the growth of connective tissue in the defect and it has osteoconductive properties. Addition of water to calcium sulfate hemihydrate produces exothermically the dihydrate salt $CaSO_4 \cdot 2H_2O$, gypsum. The as obtained product is a paste with good handling properties and mouldability which becomes a hard cement in a matter of minutes. In contact with body fluids, gypsum forms calcium phosphate deposits responsible for conducting bone formation. Despite its efficiency, the applications are limited by brittleness, and rapidity of *in vivo* resorption. Calcium sulfate + polymer systems in which the salt is encapsulated in a polymeric biodegradable and biocompatible matrix, have been developed in order to retain the structural integrity and to decrease the bioresorption rate: thus poly(ε-caprolactone), a polyester currently used for resorbable sutures, drug delivery systems and bone graft substitutes, has been used to manufacture said composites(Gomez d'Ayala, De Rosa, Laurienzo, & Malinconico, 2007). As an alternative, encapsulation of calcium sulfate in hydrogels based on blends of biocompatible polysaccharides yields a cement with good mechanical properties and slow resorption rate.

7. Osteogenic differentiation with the aid of dexamethasone

Mesenchymal stem cells are a valuable therapeutic tool in tissue engineering because they proliferate and differentiate into distinct cellular phenotypes, such as osteoblasts, chondrocytes, adipocytes and muscle cells. They might obviate to certain drawbacks of current scaffolds, such as the difficulty to seed cells deep into the scaffold, and inability for injection in minimally invasive surgery. Mesenchymal stem cells have been initially identified in bone marrow as non-hematopoietic stem cells, called bone marrow-derived stem cells. The adipose tissue, often removed during plastic surgery, might become an alternative source of mesenchymal stem cells, mainly because adipose-derived stem cells can be extracted from adipose tissue isolates in large quantities, potentially eliminating the need for *in vitro* expansion. Some reports on their use are controversial, however recent works such as that by Zhao, Weir, & Xu (2010) denote progress in their applications. In an injectable and mechanically strong stem cell composite for bone regeneration made of calcium phosphate cement and hydrogel microbeads encapsulating human umbilical cord mesenchymal stem cells, viability after injection matched that in hydrogel without cement or without injection. Mechanical properties of the composite matched the reported values of cancellous bone, and were much higher than previous injectable polymeric and hydrogel carriers. In the injectable composites osteodifferentiation took place, yielding synthesized bone inorganics, high alkaline phosphatase, osteocalcin, collagen type I, and osterix gene expressions (at 7 d they were 50–70 fold higher than at 1 d). Said injectable stem cell composite with load-bearing capability was deemed suitable to enhance bone regeneration in minimally-invasive orthopedic surgery (Zhao et al., 2010).

Dexamethasone, an anti-inflammatory glucocorticoid commonly used as an inducer of osteoblast differentiation *in vitro*, was impregnated in chitosan scaffolds using supercritical fluid technology, in order to improve the impregnation process, that in aqueous systems is jeopardized by the high viscosity of the chitosan solution and the low solubility of dexamethasone in water. Impregnation using supercritical fluid technology has proven to be feasible when the drug is soluble in carbon dioxide and the polymer can be swollen by the supercritical fluid; a pure product, free of residual solvents is obtained, because the only solvent present is the volatile carbon dioxide. Supercritical fluids, especially supercritical carbon dioxide, have prime roles in the development of clean processes for the preparation of drug-loaded polymeric supports. In this context, chitosan sponges were prepared from a 4% solution of chitosan in 2% acetic acid, contained into cylindrical moulds that were frozen at −80 °C and lyophilised. The scaffolds were neutralized with 0.1 M NaOH and washed with water, to be frozen and lyophilised again, and then they were impregnated with dexamethasone at 8.0–14.0 MPa and 35–55 °C. The highest loading was achieved at 8.0 MPa and 35 °C, thus supercritical fluid impregnation proved to be useful in this area (Duarte, Mano, & Reis, 2009).

Protracted addition of dexamethasone promotes osteoblastic differentiation *in vitro* partly by inhibiting gelatinase and by suppressing inflammatory cytokines which result in increased cell attachment and cell cycle exit. Confluent bone marrow stromal cells were cultured for 3 weeks with 16% fetal bovine serum, ascorbate-2-phosphate and disodium β-glycerophosphate, in the absence or presence of dexamethasone. Dexamethasone slowed cell division, stimulated alkaline phosphatase activity and enhanced matrix mineralization. Added chitosan particles accumulated intra- and extracellularly and, while not affecting most osteogenic features, they inhibited osteocalcin release to the media and interfered with mineralized matrix deposition. Interestingly, dexamethasone promoted cell attachment and suppressed the release and activation of matrix metalloprotease-2. While chitosan particles had no effect on the release of angiogenic factors, dexamethasone significantly inhibited the release of vascular endothelial growth factor, granulocyte-macrophage colony stimulating factor, tumor necrosis factor-α, interleukins 1 β, 4, 6, and 10, and a host of other inflammatory factors that were constitutively secreted by the bone marrow stromal cells. These results demonstrate that chitosan particles alone are not sufficient to promote osteoblast differentiation of bone marrow stromal cells *in vitro*, and are indicative

of an indirect mechanism of chitosan in promoting osteogenesis *in vivo*. In this context, nanocarriers that possess high cellular uptake efficiency to deliver and target drugs may deserve investigations, since they can allow modulation of the cellular functions in an effective manner ex vivo, and maintain the cellular phenotype *in vivo* upon re-implantation. The effect of dexamethasone-loaded carboxymethyl chitosan + poly(amidoamine) dendrimer nanoparticles on the proliferation and osteogenic differentiation of rat bone marrow stromal cells *in vitro* has been studied with the aid of O-carboxymethyl chitosan. The stromal cells seeded onto the surface of hydroxyapatite scaffolds differentiated into osteoblasts when cultured in the presence of dexamethasone-loaded nanoparticles, and enhanced osteogenesis by increasing the alkaline phosphatase activity and mineralization of the extra-cellular matrix. The pre-incubation of stem cells with these kinds of nanoparticles allowed the delivery of dexamethasone inside the cells and influenced their fate (Chen & Park, 2003; Guzman-Morales et al., 2009; Oliveira et al., 2009).

The development of novel strategies that stimulate stem cells to become osteoblasts *in vitro* and *in vivo*, and that provide a more effective treatment route with diminished complications seems to attract attention for further exploitation. Single or co-cultures of osteoblasts and osteoclasts (used at a ratio of 1:100 osteoblast:osteoclasts) were made on vapour stabilised silk fibroin, methanol stabilised silk fibroin, chitosan and poly(L-lactic acid) films for 10 days. Vapour stabilised silk fibroin, methanol stabilised silk fibroin and chitosan all support the growth of osteoblasts and osteoclasts in both single and co-cultures. Poly(L-lactic acid) showed poor osteoclast differentiation in both single and co-cultures but supported osteoblast attachment and proliferation. Both silk fibroin materials showed sign of early degradation in the 10-day period, but very little change was seen in chitosan and poly(L-lactic acid) samples. This co-culture approach for bone tissue engineering is possible if scaffolds are manufactured from silk fibroin or chitosan (Jones, Motta, Marshall, El Haj, & Cartmell, 2009).

8. Bone morphogenetic proteins

Bone morphogenetic proteins are currently approved for spinal fusion, tibial fracture repair, and maxillofacial bone regeneration. However, pleiotropism, paradoxical activities on precursor cells, and unexpected side effects at local and ectopic sites may limit their usage. An example of alternative osteoinductive factors that provide more bone-specific activities with fewer adverse effects is Nell-1 [Nel-like molecule-1; Nel (a protein highly expressed in neural tissue encoding epidermal growth factor like domain)] an osteogenic protein believed to specifically target cells committed to the osteogenic lineage. Haidar et al. reviewed a representative selection of materials suitable as carriers of rh-bone morphogenetic protein-2 and -7, and delivery systems ranging from simple nanoparticles to complex 3-D scaffolds in sites of orthopaedic and craniofacial bone regeneration and repair (Lee, Li, et al., 2009; Haidar, Hamdy, & Tabrizian, 2009).

Bone morphogenetic protein-6-loaded chitosan scaffolds enhanced the osteoblastic characteristics of MC3T3-E1 cells. In fact, they supported proliferation of said mouse osteogenic cells in a similar pattern as the unloaded chitosan scaffolds and as the chitosan scaffolds with free factor. Extracellular matrix synthesis and the levels of alkaline phosphatase and osteocalcin were higher in bone morphogenetic protein-6-loaded chitosan scaffold group than in the other groups. In addition, said loaded scaffolds showed strong staining in mineralization assays. These findings suggest that also the bone morphogenetic protein-6-loaded chitosan scaffold supports the functions of the osteoblastic cells (Akman, Tigli, Gumusderelioglu, & Nohutcu, 2010).

Chitosan oligomers (1400 Da) and high molecular weight chitosan were comparatively studied in terms of physical and biological characteristics. Both adipose and marrow stem cells preferred to attach on chitooligomer film than chitosan film with 6–7 times larger cell areas. Numbers of both stem cells proliferated on chitooligomer film were approximately 3-fold higher than those on chitosan film. In addition, chitooligomer film enhanced osteogenic differentiating, based on alkaline phosphatase activity and calcium deposition. Therefore, for the growth and osteogenic differentiation of stem cells, the chitooligomer was a more favorable material than high molecular weight chitosan (Ratanavaraporn, Kanokpanont, Tabata, & Damrongsakkul, 2009). As it was the case for osteoblasts, osteogenic progenitor cells were recruited and induced to form large numbers of colonies in the presence of chitosan, indicative of promotion of differentiation (Kim, Park, Kwon, Baik, & Cho, 2002; Park et al., 2005).

The delivery strategies for bone morphogenetic proteins were reviewed by Kirker-Head (2000) but no mention of chitosan can be found in that article. Today, however, a relatively large number of experimental works make use of chitosans for the delivery of human and recombinant bone morphogenetic proteins that induce differentiation of multipotential mesenchymal cells, and pluripotent murine stem cells.

The morphogenetic protein-2 has been widely used as an effective growth factor because it has a positive effect in every step of bone regeneration. Transforming growth factor-β-2 alone is not effective enough in bone regeneration, and combining the two factors does not lead to a better bone regeneration because no synergism takes place (Abarrategi, Civantos, Ramos, Casado, & Lopez-Lacomba, 2008; Canter et al., 2010; Issa et al., 2008; Lee et al., 2002). However, large amounts of bone morphogenetic protein-2 are required to induce new bone and the resulting side effects limit its clinical application. Sulfated polysaccharides, such as heparin and heparan sulfate have been found to modulate bone morphogenetic protein-2 bioactivity and play pivotal roles in bone metabolism. Several sulfated chitosans were synthesized by regioselective reactions firstly. Using C2C12 myoblast cells as *in vitro* models, the enhanced bioactivity of bone morphogenetic protein-2 was attributed primarily to the stimulation by 6-O-sulfated chitosan, while 2-N-sulfate group had less activation. A low dose of 2-N,6-O-sulfated chitosan showed significant enhancement on the alkaline phosphatase activity and the mineralization formation induced by bone morphogenetic protein-2, as well as the expression of alkaline phosphatase and osteocalcin mRNA. Dose-dependent effects on bone morphogenetic protein-2 bioactivity were observed in both sulfated chitosan and heparin. Compared with native heparin, the sulfated chitosan showed much stronger simultaneous effects on the bone morphogenetic protein-2 bioactivity at low dose. The bone morphogenetic protein-2 ligand bound to its receptor was enhanced by low dose of sulfated chitosan, whereas weakened by increasing amounts. Furthermore, simultaneous administration of bone morphogenetic protein-2 and sulfated chitosan *in vivo* dose-dependently induced larger amounts of ectopic bone formation compared with the factor alone. These findings indicate that sulfated chitosan is a potent enhancer of bioactivity (Zhou et al., 2009).To develop a composite capable of releasing bone morphogenetic protein-2-derived synthetic peptide, porous poly(lactic acid) + chitosan microspheres composites containing different quantities of chitosan microspheres were prepared by a thermally induced phase separation method. FTIR analysis revealed hydrogen bonds between the poly(lactic acid) and chitosan component. Introduction of less than 30% chitosan microspheres [on poly(lactic acid) weight basis] did not remarkably affect the morphology and porosity of the poly(lactic acid) + chitosan microspheres composites. The compressive strength increased from 0.48 to 0.66 MPa, while the compressive modulus increased

from 7.29 to 8.23 MPa as the microsphere contents increased to 50%. The dissolution of chitosan was preferential than poly(lactic acid) matrix and the inclusion of chitosan microspheres could neutralize the acidity of poly(lactic acid) degradation products. The release of the synthetic peptide was controlled by the degradation of poly(lactic acid) (Niu, Feng, Wang, Guo, & Zheng, 2009). Chondrocytes proliferated and secreted extracellular matrix at the same concentration as in the control. As a further step, the recombinant human bone morphogenetic protein-2 was encapsulated in poly(lactide-co-glycolide) biodegradable microspheres, which were then dispersed in a chitosan + collagen composite scaffold. The effect of rh-bone morphogenetic protein-2 encapsulated scaffolds on enhancing bone formation through implantation in canine mandibles was defined upon histological examination of the regenerated bone after 4 weeks of implantation. Due to PLGA microspheres, said scaffold exhibited lower porosity values and swelling rate, but higher release than control. Bone density, bone/implant contact, and bone-fill values *in vivo* demonstrated that the composite scaffold induced bone regeneration more quickly and that it was promptly replaced by new bone. This sustained carrier composite based on microspheres was more effective in inducing implant osteointegration (Shi, Cheng, et al., 2009; Shi, Neoh, et al., 2009; Tan, Wu, Lao, & Gao, 2009).

In agreement with those observations, the addition of type I collagen to chitosan gels dramatically increased cell spreading, and h-bone marrow stromal cells became spindle-shaped and proliferated more than in pure chitosan. In chitosan + collagen composites, higher collagen content did not lead to increased DNA content of gels suggesting that the cell number in these materials was similar. The advantage was higher expression of osteogenic genes, increased alkaline phosphatase and higher calcium content. Addition of collagen also resulted in a stiffer material, relative to pure chitosan. The osteogenic differentiation of h-bone marrow stromal cells was correlated to the chitosan content in the composites in agreement with previous studies reporting that rat progenitor cells deposit calcium when embedded in pure chitosan gels initiated by β-glycerophosphate addition (Arpornmaeklong, Pripatnanont, & Suwatwirote, 2008; Wang & Stegemann, 2010).

Because electrospinning makes available thin (30–40 μm) mats of chitosan fibres with diameters typically close to 125 nm, their suitability for osteoblast attachments and growth has been investigated, and the results were compared with the corresponding solvent-cast films. Whilst both mats and films supported the attachment and, at the same time, promoted the largest increase in the viability of keratinocytes, they showed cytostatic property towards both osteoblast-like cells and fibroblasts, despite the convincingly good attachment of osteoblast-like cells on the surfaces. The observed incapacity of osteoblasts to proliferate despite the favorable biocompatibility of the materials emphasizes the role of both surface area and porosity of the support, that in the mentioned case were possibly far from optimal values. It should be remarked however that these results are at variance with previous ones obtained by another group with two chitosans in film form, where the osteoblast attachment at 1 h was significantly greater than for fibroblasts. At 24 h, levels of cell attachment for fibroblasts increased and became similar to those in osteoblast cultures at 1 and 24 h. Fibroblasts showed heterogeneous population of round and semi-spread cells, but in comparison, osteoblasts displayed phenotypes that were well spread with a developed cytoskeleton. At the present time, the experimental data with electrospun chitosans are too few for attempting an interpretation (Fakhry, Schneider, Zaharias, & Senel, 2004; Sangsanoh et al., 2010).

Double-face scaffolds based on hydroxyapatite dispersed into chitosan crosslinked with glutaraldehyde were developed based on the optimization of both polymeric and composite scaffolds: tomography was carried out to accurately quantify porosity, interconnectivity, ceramic content, particle and pore size. The scaffolds were highly interconnected, presented the ideal pore size range to be morphometrically suitable for the proposed applications, and were mechanically stable in the wet state even under dynamic compression. The obtained elastic modulus was 4.21 ± 1.04, 7.98 ± 1.77 and 6.26 ± 1.04 MPa for polymeric, composite and bilayered scaffolds, respectively. Bioactivity studies using a simulated body fluid and a simulated synovial fluid were conducted in order to assure that the polymeric component for chondrogenic part would not mineralize, as confirmed by scanning electron microscopy, inductively coupled plasma-optical emission spectroscopy and energy-dispersive spectroscopy for different immersion periods. The assays were carried out also under dynamic conditions using, for this purpose, a specifically designed double-chamber bioreactor, aiming at a future osteochondral application. It was concluded that chitosan-based bilayered scaffolds produced by particle aggregation overcome any risk of delamination of both polymeric and composite parts designed for mechanically stable chondrogenic and osteogenic components, respectively. Other authors are in favor of the application of a single-phase material, endowed with gradients of molecular, structural and functional properties, such as, for example, a single phase silk-based scaffold functionalized by covalently binding growth factors with opposing gradients of a chondrogenic factor (IGF-I) and an osteogenic factor (bone morphogenetic protein-2) for tissue engineering of osteochondral grafts. The opposite gradients of these two different growth factors in the same scaffold intended to mimic the most suitable concentration of IGF-I for cartilage at one end and the most suitable concentration of bone morphogenetic protein-2 for bone at the other end (Malafaya & Reis, 2009; Vunjak-Novakovic, Meinel, Altman, & Kaplan, 2005).

9. Conclusion

Chitosan respects the physiological bone formation and healing processes, and most importantly it enhances favorably the biochemical responses, thanks to its inherent immunostimulating properties and susceptibility to lysozyme. Bone healing involves a sequence of events that should not be disturbed by the presence of a composite or scaffold. At the time of a fracture, the disruption of bone architecture and vascular network results in loss of mechanical stability and local decrease in oxygen and nutrients. The inflammatory response is accompanied with the activation of macrophages and infiltration of platelets that release various cytokines, which probably play a role in the initiation of the repair process by acting on various cells: post-fracture periosteal osteoprogenitor cells and osteoblasts differentiate to produce new bone. This process involves fibroblast growth factors and bone morphogenetic proteins. To provide crucial nutrient supplies to the cells, new blood vessels develop into the fracture callus. The matrix composed of various collagen isotypes develops, which may be important for presenting cytokines to receptive cells.

The chemical and technological versatility of chitosan enables researchers to prepare elaborated composites for the purpose indicated in the previous paragraph: for example, the research works on bone regeneration with the aid of bone cements have recently become more refined in terms of the effects of chitosan composites on the cells involved in the healing process.

The use of nano-hydroxyapatite as well as other inorganics in conjunction with variously modified chitosans is greatly contributing to the advancement and exploitation of chitosan composites for bone healing. With the advent of nanotechnology the applications of fairly non-toxic nanocrystalline hydroxyapatite extends from bone repair and augmentation to the delivery of drugs, growth factors and genetic material to the bone: for this purpose, particles of uniform size with controlled morphology can be manufactured

by using macromolecules as templates. A number of advantages have become evident, particularly when nano-hydroxyapatite is crystallized using biomimetic methods, or when the biopolymers are submitted to biomineralization. The hydroxyapatite nanoparticles influence favorably the morphology of attached cells, as a consequence of the adsorption of extracellular matrix proteins from serum, that in turn bind osteoblast precursors. Thus, an additional peculiarity of chitosan emerging from the most recent studies, is the capacity to influence both the mineralization and the cell activity.

When a bone fracture requires an orthopedic implant, it is important to obtain durable integration between the metallic support and healthy bone: titanium alloys electrolytically coated with apatite and treated with chitosan exhibit good bone marrow stromal cell attachment and increased osteoblast proliferation. The current technology permits to manufacture chitosan microparticles coated with bone-like mineral layers, useful to assist bone growth: in particular, chitosan + silica composites are being manufactured in various physical forms, including injectable gels.

Chitosan, N-carboxymethyl chitosan, fibroin and poly(L-lactic acid) are at the basis of new strategies useful to stimulate stem cells to become osteoblasts, and to make co-cultures of osteoblasts and osteoclasts. Thanks to chitosan and to sulfated chitosan, important advances have been made in the field of the delivery of human and recombinant bone morphogenetic proteins, in particular the morphogenetic protein-2 that exhibits a positive effect in every step of the bone regeneration.

As shown in this review article, that testifies the importance of the body of knowledge today available on the title topic, the advances made in histology, cell culture, and cytology are matched by the contributions from material chemistry. Nevertheless, it appears that even most significant observations such as the synergistic activity of chitosan with inorganic compounds in influencing cell behavior are still affected by empiricism in the chemical preparations. On the other hand, rigorous chemical control over the preparation and the characterization of chitins and chitosans, and the refinement of certain techniques such as electrospinning, porosity optimization, and nanocrystalline chitosan preparation, will certainly lead to important advances toward full exploitation of the growing valuable knowledge about chitosan composites.

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References

Abarrategi, A., Civantos, A., Ramos, V., Casado, J. V. S., & Lopez-Lacomba, J. L. (2008). Chitosan film as rhBMP2 carrier: delivery properties for bone tissue application. *Biomacromolecules*, *9*, 711–718.

Aday, S., & Gumusderelioglu, M. (2010). Bone-like apatite-coated chitosan scaffolds: characterization and osteoblastic activity. *Polymer Composites*, *31*, 1418–1426.

Akman, A. C., Tigli, R. S., Gumusderelioglu, M., & Nohutcu, R. M. (2010). Bone morphogenetic protein-6-loaded chitosan scaffolds enhance the osteoblastic characteristics of MC3T3-E1 cells. *Artificial Organs*, *34*, 65–74.

Arpornmaeklong, P., Pripatnanont, P., & Suwatwirote, N. (2008). Properties of chitosan-collagen sponges and osteogenic differentiation of rat-bone-marrow stromal cells. *International Journal of Oral and Maxillofacial Surgery*, *37*, 357–366.

Borah, C., Scott, G., & Wortham, K. (1992). Bone induction by chitosan. In C. J. Brine, P. A. Sandford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 47–53). Amsterdam: Elsevier.

Boynuegri, D., Ozcan, G., Senel, S., Uc, D., Uraz, A., Ogus, E., et al. (2009). Clinical and radiographic evaluations of chitosan gel in periodontal intraosseous defects: a pilot study. *Journal of Biomedical Materials Research*, *90-B*, 461–466.

Bumgardner, J. D., Wiser, R., Elder, S. H., Jouett, R., Yang, Y., & Ong, J. L. (2003). Contact angle, protein adsorption and osteoblast precursor cell attachment to chitosan coatings bonded to titanium. *Journal of Biomaterial Science Polymer Edition*, *14*, 1401–1409.

Bumgardner, J. D, Wiser, R., Gerard, D., Bergin, P., Chestnutt, B., Marin, M., et al. (2003). Chitosan: potential use as a bioactive coating for orthopaedic and craniofacial/dental implants. *Journal of Biomaterial Science Polymer Edition*, *14*, 423–438.

Calandrelli, L., Della Ragione, F., d'Ayala, G. G., Laurienzo, P., Malinconico, M., & Oliva, A. (2010). *Biodegradable composites for bone regeneration. In Bone regeneration: Growth factors, augmentation procedures and tissue engineering applications*. Hauppauge, USA: Nova Science Publishers Inc., pp. 1–57.

Canter, H. I., Vargel, I., Korkusuz, P., Oner, F., Gungorduk, D. B., Cil, B., et al. (2010). Effect of use of slow release of bone morphogenetic protein-2 and transforming growth factor-beta-2 in a chitosan gel matrix on cranial bone graft survival in experimental cranial critical size defect model. *Annals of Plastic Surgery*, *64*, 342–350.

Castagnino, E., Ottaviani, M. F., Cangiotti, M., Morelli, M., Casettari, L., & Muzzarelli, R. A. A. (2008). Radical scavenging activity of 5-methylpyrrolidinone chitosan and dibutyryl chitin. *Carbohydrate Polymers*, *74*, 640–647.

Chang, S. J., Kuo, S. M., & Lan, C. W. (2009). Evaluation of chitosan/$CaSO_4$/platelet-rich plasma microsphere composites as alveolus osteogenesis material. *Biomedical Engineering – Applications Basis Communications*, *21*, 115–122.

Chen, X. G., & Park, H. J. (2003). Chemical characteristics of O-carboxymethyl chitosans related to the preparation conditions. *Carbohydrate Polymers*, *53*, 355–359.

Chen, F., Wang, Z. C., & Lin, C. J. (2002). Preparation and characterization of nano-sized hydroxyapatite particles and hydroxyapatite/chitosan nano-composite for use in biomedical materials. *Materials Letters*, *57*, 858–861.

Cheng, X. M., Li, Y. B., Zuo, Y., Zhang, L., Li, J. D., & Wang, H. A. (2009). Properties and in vitro biological evaluation of nano-hydroxyapatite/chitosan membranes for bone guided regeneration. *Materials Science and Engineering, 29C*, 29–35.

Chen, J. D., Wang, Y. J., & Chen, X. F. (2009). Designing nano-hydroxyapatite in the polyelectrolyte complex macroporous scaffold based on chitosan and hyaluronic acid. *Materials Research*, 1252–1254, 610-613 (Pts 1–2).

Chesnutt, B. M., Viano, A. M., Yuan, Y. L., Yang, Y. Z., Guda, T., Appleford, M. R., et al. (2009). Design and characterization of a novel chitosan/nanocrystalline calcium phosphate composite scaffold for bone regeneration. *Journal of Biomedical Materials Research*, *88A*, 491–502.

Chiang, T. Y., Ho, C. C., Chen, D. C. H., Lai, M. H., & Ding, S. J. (2010). Physicochemical properties and biocompatibility of chitosan oligosaccharide/gelatin/calcium phosphate hybrid cements. *Materials Chemistry and Physics*, *120*, 282–288.

Chua, P. H., Neoh, K. G., Kang, E. T., & Wang, W. (2008). Surface functionalization of titanium with hyaluronic acid /chitosan polyelectrolyte multilayers and RGD for promoting osteoblast functions and inhibiting bacterial adhesion. *Biomaterials*, *29*, 1412–1421.

Couto, D. S., Hong, Z. K., & Mano, J. F. (2009). Development of bioactive and biodegradable chitosan-based injectable systems containing bioactive glass nanoparticles. *Acta Biomaterialia*, *5*, 115–123.

Cui, X., Zhang, B., Wang, Y., & Gao, Y. L. (2008). Effects of chitosan-coated pressed calcium sulfate pellet combined with recombinant human bone morphogenetic protein 2 on restoration of segmental bone defect. *Journal of Craniofacial Surgery*, *19*, 459–465.

Danilchenko, S. N., Kalinkevich, O. V., Kuznetsov, V. N., Kalinkevich, A. N., Kalinichenko, T. G., Poddubny, I. N., et al. (2010). Thermal transformations of the mineral component of composite biomaterials based on chitosan and apatite. *Crystal Research and Technology*, *45*, 685–691.

Degim, Z. (2008). Use of microparticulate systems to accelerate skin wound healing. *Journal of Drug Targeting*, *16*, 437–448.

Dorozhkin, S. V., & Epple, M. (2002). Biological and medical significance of calcium phosphates. *Angewandte Chemie International Edition*, *41*, 3130–3146.

Duarte, A. R. C., Mano, J. F., & Reis, R. L. (2009). Preparation of chitosan scaffolds loaded with dexamethasone for tissue engineering applications using supercritical fluid technology. *European Polymer Journal*, *45*, 141–148.

Dupoirieux, L., Pourquier, D., Picot, M. C., & Neves, M. (2001). Comparative study of three different membranes for guided bone regeneration of rat cranial defects. *International Journal of Oral Maxillofacial Surgery*, *30*, 58–62.

Fakhry, A., Schneider, G. B., Zaharias, R., & Senel, S. (2004). Chitosan supports the initial attachment and spreading of osteoblasts preferentially over fibroblasts. *Biomaterials*, *25*, 2075–2079.

Fan, H. L., Wang, L. L., Zhao, K. K., Li, N., Shi, Z. J., Ge, Z. G., et al. (2010). Fabrication, mechanical properties, and biocompatibility of graphene-reinforced chitosan composites. *Biomacromolecules*, *11*, 2345–2351.

Geffre, C. P., Ochoa, J., Margolis, D. S., & Szivek, J. A. (2010). Evaluation of the osteogenic performance of calcium phosphate-chitosan bone fillers. *Journal of Investigative Surgery*, *23*, 134–141.

Gomez d'Ayala, G., De Rosa, A., Laurienzo, P., & Malinconico, M. (2007). Development of a new calcium sulphate-based composite using alginate and chemically modified chitosan for bone regeneration. *Journal of Biomedical Materials Research*, *81A*, 811–820.

Greene, A. H., Bumgardner, J. D., Yang, Y., Moseley, J., & Haggard, W. O. (2008). Chitosan-coated stainless steel screws for fixation in contaminated fractures. *Clinical Orthopaedics and Related Research*, *466*, 1699–1704.

Guzman-Morales, J., El-Gabalawy, H., Pham, M. H., Tran-Khanh, N., McKee, M. D., Wu, W., et al. (2009). Effect of chitosan particles and dexamethasone on human bone marrow stromal cell osteogenesis and angiogenic factor secretion. *Bone*, *45*, 617–626.

Haidar, Z. S., Hamdy, R. C., & Tabrizian, M. (2009). Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair Part B: Delivery sys-

tems for BMPs in orthopaedic and craniofacial tissue engineering. *Biotechnology Letters*, *31*, 1825–1835.
Issa, J. P. M., DoNascimento, C., Bentley, M. V. L. B., DelBel, E. A., Iyomasa, M. M., Sebald, W., et al. (2008). Bone repair in rat mandible by rhBMP-2 associated with two carriers. *Micron*, *39*, 373–379.
Ito, M. (1991). In vitro properties of a chitosan bonded bone-filling paste. *Biomaterials*, *12*, 41–45.
Ito, M., & Hidaka, Y. (1997). A chitosan bonded hydroxyapatite bone filling material. In R. A. A. Muzzarelli (Ed.), *Chitin handbook* (pp. 373–389). Italy: Atec.
Jayakumar, R., Chennazhi, K. P., Muzzarelli, R. A. A., Tamura, H., Nair, S. V., & Selvamurugan, N. (2010). Chitosan conjugated DNA nanoparticles in gene therapy. *Carbohydrate Polymers*, *79*, 1–8.
Jayasuriya, A. C., & Kibbe, S. (2010). Rapid biomineralization of chitosan microparticles to apply in bone regeneration. *Journal of Materials Science – Materials in Medicine*, *21*, 393–398.
Jollès, P., & Muzzarelli, R. A. A (Eds.). (1999). *Chitin and chitinases*. Basel: Birkhauser Verlag.
Jones, G. L., Motta, A., Marshall, M. J., El Haj, A. J., & Cartmell, S. H. (2009). Osteoblast /osteoclast co-cultures on silk fibroin, chitosan and PLLA films. *Biomaterials*, *30*, 5376–5384.
Kang, Y. M., Kim, K. H., Seol, Y. J., & Rhee, S. H. (2009). Evaluations of osteogenic and osteoconductive properties of a non-woven silica gel fabric made by the electrospinning method. *Acta Biomaterialia*, *5*, 462–469.
Keong, L. C., & Halim, A. S. (2009). In vitro models in biocompatibility assessment for biomedical grade chitosan derivatives in wound management. *International Journal of Molecular Sciences*, *10*, 1300–1313.
Kim, I. S., Park, J. W., Kwon, I. C., Baik, B. S., & Cho, B. C. (2002). Role of BMP, betaigh3, and chitosan in early bone consolidation in distraction osteogenesis in a dog model. *Plastic and Reconstruction Surgery*, *109*, 1966–1977.
Kirker-Head, C. A. (2000). Potential applications and delivery strategies for bone morphogenetic proteins. *Advanced Drug Delivery Reviews*, *43*, 65–92.
Klokkevold, P. R., Vandemark, L., Kennedy, E. B., & Bernard, G. W. (1996). Osteogenesis enhanced by chitosan in vitro. *Journal of Periodontology*, *67*, 1170–1177.
Kostopoulos, L., & Karring, T. (1994). Guided bone regeneration in mandibular defects in rats using a bioresorbable polymer. *Clinical Oral Implants Research*, *5*, 666–674.
Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews*, *104*, 6017–6084.
Kurita, K. (2006). Chitin and chitosan: functional biopolymers from marine crustaceans. *Marine Biotechnology*, *8*, 203–226.
Lee, E. J., Jun, S. H., Kim, H. E., Kim, H. W., Koh, Y. H., & Jang, J. H. (2010). Silica xerogel-chitosan nano-hybrids for use as drug eluting bone replacement. *Journal of Materials Science – Materials in Medicine*, *21*, 207–214.
Lee, E. J., Shin, D. S., Kim, H. E., Kim, H. W., Koh, Y. H., & Jang, J. H. (2009). Membrane of hybrid chitosan-silica xerogel for guided bone regeneration. *Biomaterials*, *30*, 743–750.
Lee, J. Y., Nam, S. H., Im, S. Y., Park, Y. J., Lee, Y. M., Seol, Y. J., et al. (2002). Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials. *Journal of Controlled Release*, *78*, 187–197.
Lee, M., Li, W. M., Siu, R. K., Whang, J., Zhang, X. L., Soo, C., et al. (2009). Biomimetic apatite-coated alginate/chitosan microparticles as osteogenic protein carriers. *Biomaterials*, *30*, 6094–6101.
Lee, S. H., Kim, B. J., Shin, S. H., Kim, H. S., Kim, K. C., Kim, C. H., et al. (2009). Guided bone regeneration effect by chitosan/hydroxyapatite membrane on repair of rat calvarial defect. *Tissue Engineering and Regenerative Medicine*, *6*, 916–923.
Lee, Y. K., & Mooney, D. J. (2001). Hydrogels for tissue enigineering. *Chemical Reviews*, *101*, 1869–1879.
Leroux, L., Hatim, Z., Freche, M., & Lacout, J. L. (1999). Effects of various adjuvants lactic acid, glycerol, and chitosan on the injectability of a calcium phosphate cement. *Bone*, *5*, 31s–34s.
Li, B., Wang, X. B., Ma, J. H., & Huang, L. N. (2009). Preparation of phosphorylated chitosan/chitosan/hydroxyapatite composites by co-precipitation method. *Multi-Functional Materials and Structures II*, 401–404, 79–82 (Pt 1–2).
Li, J. J., Dou, Y., Yang, J., Yin, Y. J., Zhang, H., Yao, F. L., et al. (2009). Surface characterization and biocompatibility of micro- and nano-hydroxyapatite/chitosan-gelatin network films. *Materials Science and Engineering*, *29C*, 1207–1215.
Li, L. H., Kommareddy, K. P., Pilz, C., Zhou, C. R., Fratzl, P., & Manjubala, I. (2010). In vitro bioactivity of bioresorbable porous polymeric scaffolds incorporating hydroxyapatite microspheres. *Acta Biomaterialia*, *6*, 2525–2531.
Li, Z., Li, Y. B., Yi, Z., Lan, W., & Jansen, J. A. (2010). In vitro and in vivo evaluation on the bioactivity of ZnO containing nano-hydroxyapatite/chitosan cement. *Journal of Biomedical Materials Research*, *93A*, 269–279.
Liao, F., Chen, Y. Y., Li, Z. B., Wang, Y. N., Shi, B., Gong, Z. C., et al. (2010). A novel bioactive three-dimensional beta-tricalcium phosphate/chitosan scaffold for periodontal tissue engineering. *Journal of Materials Science – Materials In Medicine*, *21*, 489–496.
Lim, T. Y., Wang, W., Shi, Z. L., Poh, C. K., & Neoh, K. G. (2009). Human bone marrow-derived mesenchymal stem cells and osteoblast differentiation on titanium with surface-grafted chitosan and immobilized bone morphogenetic protein-2. *Journal of Materials Science – Materials in Medicine*, *20*, 1–10.
Lopez-Perez, P. M., da Silva, R. M. P., Serra, C., Pashkuleva, I., & Reis, R. L. (2010). Surface phosphorylation of chitosan significantly improves osteoblast cell viability, attachment and proliferation. *Journal of Materials Chemistry*, *20*, 483–491.
Low, K. L, Tan, S. H., Zein, S. H. S., Roether, J. A., Mourino, V., & Boccaccini, A. R. (2010). Calcium phosphate-based composites as injectable bone substitute materials. *Journal of Biomedical Materials Research*, *94B*, 273–286.
Malafaya, P. B., & Reis, R. L. (2009). Bilayered chitosan-based scaffolds for osteochondral tissue engineering: Influence of hydroxyapatite on in vitro cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor. *Acta Biomaterialia*, *5*, 644–660.
Maniatopoulos, C., Sodek, J., & Melcher, A. (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell and Tissue Research*, *254*, 317–330.
Martins, A. M., Alves, C. M., Kasper, F. K., Mikos, A. G., & Reis, R. L. (2010). Responsive and in situ-forming chitosan scaffolds for bone tissue engineering applications: an overview of the last decade. *Journal of Materials Chemistry*, *20*, 1638–1645.
Maruyama, M., & Ito, M. (1996). In vitro properties of a chitosan-bonded self-hardening paste with hydroxyapatite granules. *Journal of Biomedical Material Research*, *32*, 527–533.
Mattioli-Belmonte, M., Biagini, G., Muzzarelli, R. A. A., Castaldini, C., Gandolfi, M. G., Krajewski, A., et al. (1995). Osteoinduction in the presence of chitosan-coated porous hydroxyapatite. *Journal of Bioactive and Compatible Polymers*, *10*, 249–257.
Mattioli-Belmonte, M., Nicoli-Aldini, N., De Benedittis, A., Sgarbi, G., Amati, S., Fini, M., et al. (1999). Morphological study of bone regeneration in the presence of 6-oxychitin. *Carbohydrate Polymers*, *40*, 23–27.
Mourino, V., & Boccaccini, A. R. (2010). Bone tissue engineering therapeutics: controlled drug delivery in three-dimensional scaffolds. *Journal of the Royal Society Interface*, *7*, 209–227.
Mourya, V. K., & Inamdar, N. N. (2008). Chitosan-modifications and applications: Opportunities galore. *Reactive and Functional Polymers*, *68*, 1013–1051.
Muzzarelli R A A (2010). Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. In *Marine Drugs, an open access journal, Marine Chitin and Chitosan issue*, Basel. ISSN 1660-3397. www.mdpi.com/journal/marinedrugs.
Muzzarelli, R. A. A. (2009a). Aspects of chitin chemistry and enzymology. In M. Paoletti, & S. Musumeci (Eds.), *Binomium chitin-chitinase: emerging issues*. Hauppauge, USA: Nova Science Publishers Inc.
Muzzarelli, R. A. A. (2009b). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*, 167–182.
Muzzarelli, R. A. A. (1977). *Chitin*. Oxford: Pergamon Press.
Muzzarelli, R. A. A., & Muzzarelli, C. (2009). Chitin and chitosan hydrogels. In G. O. Phillips, & P. A. Williams (Eds.), *Handbook of hydrocolloids*. (2nd ed., pp. 849–888). Cambridge UK: Woodhead Publishing Ltd.
Muzzarelli, R. A. A., & Muzzarelli, C. (2006). Chitosan a dietary supplement and a food technology commodity. In C. G. Biliaderis, & M. S. Izydorczyk (Eds.), *Functional food carbohydrates* (pp. 215–244). n, USA: Francis & Taylor.
Muzzarelli, R. A. A., & Muzzarelli, C. (2005a). Chitosan chemistry: relevance to the biomedical sciences. In T. Heinze (Ed.), *Advances in polymer science* (pp. 151–171). Berlin: Springer-Verlag.
Muzzarelli, R. A. A., & Muzzarelli, C. (2005b). Chitin nanofibrils. In P. K. Dutta (Ed.), *Chitin and chitosan, opportunities and challenges*. New Delhi, India: New Age International.
Muzzarelli, R. A. A., & Muzzarelli, C. (2002). Natural and artificial chitosan-inorganic composites. *Journal of Inorganic Biochemistry*, *92*, 89–94.
Muzzarelli, R. A. A., Biagini, G., DeBenedittis, A., Mengucci, P., Majni, G., & Tosi, G. (2001). Chitosan–oxychitin coatings for prosthetic materials. *Carbohydrate Polymers*, *45*, 35–41.
Muzzarelli, R. A. A., Ramos, V., Stanic, V., Dubini, B., Mattioli-Belmonte, M., Tosi, G., et al. (1998). Osteogenesis promoted by calcium phosphate dicarboxymethyl chitosan. *Carbohydrate Polymers*, *36*, 267–276.
Muzzarelli, R. A. A., Biagini, G., Mattioli-Belmonte, M., Talassi, O., Gandolfi, M. G., Solmi, R., et al. (1997). Osteoinduction by chitosan-complexed BMP: morphostructural response in an osteoporotic model. *Journal of Bioactive and Compatible Polymers*, *12*, 321–329.
Muzzarelli, R. A. A., Biagini, G., Bellardini, M., Simonelli, C., Castaldini, C., & Fratto, G. (1993). Osteoconduction exerted by N-methylpyrrolidinone chitosan used in dental surgery. *Biomaterials*, *14*, 39–43.
Muzzarelli, R. A. A., Biagini, G., Mattioli-Belmonte, M., Talassi, O., Gandolfi, M. G., Solmi, R., et al. (1993). Osteoconductive properties of methylpyrrolidinone chitosan in an animal model. *Biomaterials*, *14*, 925–929.
Muzzarelli, R. A. A., Biagini, G., Pugnaloni, A., Filippini, O., Baldassarre, V., Castaldini, C., et al. (1989). Reconstruction of parodontal tissue with chitosan. *Biomaterials*, *10*, 598–603.
Muzzarelli, R. A. A., Bicchiega, V., Biagini, G., Pugnaloni, A., & Rizzoli, R. (1992). Role of N-carboxybutyl chitosan in the repair of the meniscus. *Journal of Bioactive and Compatible Polymers*, *7*, 130–148.
Nitzsche, H., Lochmann, A., Metz, H., Hauser, A., Syrowatka, F., Hempel, E., et al. (2010). Fabrication and characterization of a biomimetic composite scaffold for bone defect repair. *Journal of Biomedical Materials Research*, *94A*, 298–307.
Niu, X., Feng, Q., Wang, M., Guo, X., & Zheng, Q. (2009). In vitro degradation and release behavior of porous poly(lactic acid) scaffolds containing chitosan microspheres as a carrier for BMP-2-derived synthetic peptide. *Polymer Degradation and Stability*, *94*, 176–182.
Oktay, E. O., Demiralp, B., Demiralp, B., Senel, S., Akman, A. C., Eratalay, K., et al. (2010). Effects of platelet-rich plasma and chitosan combination on bone regeneration in experimental rabbit cranial defects. *Journal of Oral Implantology*, *36*, 175–184.

Oliveira, J. M., Costa, S. A., Leonor, I. B., Malafaya, P. B., Mano, J. F., & Reis, R. L. (2009). Novel hydroxyapatite/carboxymethylchitosan composite scaffolds prepared through an innovative "autocatalytic" electroless coprecipitation route. *Journal of Biomedical Materials Research*, *88A*, 470–480.

Pang, X., & Zhitomirsky, I. (2007). Electrophoretic deposition of composite hydroxyapatite-chitosan coatings. *Materials Characterization*, *58*, 339–348.

Park, D. J., Choi, B. H., Zhu, S. J., Huh, J. Y., Kim, B. Y., & Lee, S. H. (2005). Injectable bone using chitosan-alginate gel/mesenchymal stem cells /BMP-2 composites. *Journal of Cranio-Maxillofacial Surgery*, *33*, 50–54.

Peter, M., Ganesh, N., Selvamurugan, N., Nair, S. V., Furuike, T., Tamura, H., et al. (2010). Preparation and characterization of chitosan–gelatin-nanohydroxyapatite composite scaffolds for tissue engineering applications. *Carbohydrate Polymers*, *80*, 687–694.

Piattelli, A., Scarano, A., Russo, P., & Matarasso, S. (1996). Evaluation of guided bone regeneration in rabbit tibia using bioresorbable and non-resorbable membranes. *Biomaterials*, *17*, 791–796.

Puppi, D., Chiellini, F., Piras, A. M., & Chiellini, E. (2010). Polymeric materials for bone and cartilage repair. *Progress in Polymer Science*, *35*, 403–440.

Qiu, Y. Z., Zhang, N., Kang, Q., An, Y. H., & Wen, X. J. (2009). Chemically modified light-curable chitosans with enhanced potential for bone tissue repair. *Journal of Biomedical Materials Research*, *89A*, 772–779.

Ratanavaraporn, J., Kanokpanont, S., Tabata, Y., & Damrongsakkul, S. (2009). Growth and osteogenic differentiation of adipose-derived and bone marrow-derived stem cells on chitosan and chitooligosaccharide films. *Carbohydrate Polymers*, *78*, 873–878.

Rinaudo, M. (2006a). Characterization and properties of some polysaccharides used as biomaterials. *Macromolecular Symposia*, *245*, 549–557.

Rinaudo, M. (2006b). Chitin and chitosan: properties and applications. *Progress in Polymer Science*, *31*, 603–632.

Rochet, N., Balaguer, T., Boukhechba, F., Laugier, J. P., Quincey, D., Goncalves, S., et al. (2009). Differentiation and activity of human preosteoclasts on chitosan enriched calcium phosphate cement. *Biomaterials*, *30*, 4260–4267.

Sahithi, K., Swetha, M., Ramasamya, K., Sriniyasan, N., & Selyamurugan, N. (2010). Polymeric composites containing carbon nanotubes for bone tissue engineering. *International Journal of Biological Macromolecules*, *46*, 281–283.

Sahoo, D., Sahoo, S., Mohanty, P., Sasmal, S., & Nayak, P. L. (2009). Chitosan: a new versatile bio-polymer for various applications. *Designed Monomers and Polymers*, *12*, 377–404.

Sangsanoh, P., Suwantong, O., Neamnark, A., Cheepsunthorn, P., Pavasant, P., & Supaphol, P. (2010). In vitro biocompatibility of electrospun and solvent-cast chitosan substrata towards Schwann, osteoblast, keratinocyte and fibroblast cells. *European Polymer Journal*, *46*, 428–440.

Sharma, S., Soni, V. P., & Bellare, J. R. (2009). Chitosan reinforced apatite-wollastonite coating by electrophoretic deposition on titanium implants. *Journal of Materials Science – Materials in Medicine*, *20*, 1427–1436.

Shi, S. S., Cheng, X. R., Wang, J. W., Zhang, W., Peng, L., & Zhang, Y. F. (2009). RhBMP-2 microspheres-loaded chitosan/collagen scaffold enhanced osseointegration: an experiment in dog. *Journal of Biomaterials Applications*, *23*, 331–346.

Shi, Z. L., Neoh, K. G., Kang, E. T., Poh, C. K., & Wang, W. (2009). Surface functionalization of titanium with carboxymethyl chitosan and immobilized bone morphogenetic protein-2 for enhanced osseointegration. *Biomacromolecules*, *10*, 1603–1611.

Shirosaki, Y., Botelho, C. M., Lopes, M. A., & Santos, J. D. (2009). Synthesis and characterization of chitosan-silicate hydrogel as resorbable vehicle for Bonelike ® bone graft. *Journal of Nanoscience and Nanotechnology*, *9*, 3714–3719.

Shirosaki, Y., Tsuru, K., Hayakawa, S., Osaka, A., Lopes, M. A., Santos, J. D., et al. (2009). Physical, chemical and in vitro biological profile of chitosan hybrid membrane as a function of organosiloxane concentration. *Acta Biomaterialia*, *5*, 346–355.

Sikorski, P., Hori, R., & Wada, M. (2009). Revisit of alpha-chitin crystal structure using high resolution X-ray diffraction data. *Biomacromolecules*, *10*, 1100–1105.

Sogias, I. A., Williams, A. C., & Khutoryanskiy, V. V. (2008). Why is chitosan mucoadhesive? *Biomacromolecules*, *9*, 1837–1842.

Song, H. Y., Rahman, A. H. M. E., & Lee, B. T. (2009). Fabrication of calcium phosphate-calcium sulfate injectable bone substitute using chitosan and citric acid. *Journal of Materials Science – Materials In Medicine*, *20*, 935–941.

Sun, F., Lim, B. K., Ryu, S. C., Lee, D., & Lee, J. (2010). Preparation of multi-layered film of hydroxyapatite and chitosan. *Materials Science and Engineering*, *C-30*, 789–794.

Suphasiriroj, W., Yotnuengnit, P., Surarit, R., & Pichyangkura, R. (2009). The fundamental parameters of chitosan in polymer scaffolds affecting osteoblasts MC3T3-E1. *Journal of Materials Science – Materials in Medicine*, *20*, 309–320.

Swetha, M., Sahithi, K., Moorthi, A., Srinivasan, N., Ramasamy, K., & Selvamurugan, N. (2010). Biocomposites containing natural polymers and hydroxyapatite for bone tissue engineering. *International Journal of Biological Macromolecules*, *47*, 1–4.

Tan, H. P., Wu, J. D., Lao, L. H., & Gao, C. Y. (2009). Gelatin/chitosan /hyaluronan scaffold integrated with PLGA microspheres for cartilage tissue engineering. *Acta Biomaterialia*, *5*, 328–337.

Teng, S. H., Lee, E. J., Yoon, B. H., Shin, D. S., Kim, H. E., & Oh, J. S. (2009). Chitosan/nanohydroxyapatite composite membranes via dynamic filtration for guided bone regeneration. *Journal of Biomedical Materials Research*, *88A*, 569–580.

Thein-Han, W. W., & Misra, R. D. K. (2009 b). Biomimetic chitosan-nanohydroxyapatite composite scaffolds for bone tissue engineering. *Acta Biomaterialia*, *5*, 1182–1197.

Thein-Han, W. W., & Misra, R. D. K. (2009). Three-dimensional chitosan–nanohydroxyapatite composite scaffolds for bone tissue engineering. *JOM*, *61*, 41–44.

Tigli, R. S., Ghosh, S., Laha, M. M., Shevde, N. K., Daheron, L., Gimble, J., et al. (2009). Comparative chondrogenesis of human cell sources in 3D scaffolds. *Journal of Tissue Engineering and Regenerative Medicine*, *3*, 348–360.

Varlamov, V. P., Bykova, V. M., Vikhoreva, G. A., Lopatin, S. A., & Nemtsev, S. V. (Eds.). (2003). *Modern perspectives in chitin and chitosan studies*. Moscow: Vniro.

Venugopal, J., Prabhakaran, M. P., Zhang, Y. Z., Low, S., Choon, A. T., & Ramakrishna, S. (2010). Biomimetic hydroxyapatite-containing composite nanofibrous substrates for bone tissue engineering. *Philosophical Transactions of The Royal Society*, *368A*, 2065–2081.

Vunjak-Novakovic, G., Meinel, L., Altman, G., & Kaplan, D. (2005). Bioreactor cultivation of osteochondral grafts. *Orthodontics and Craniofacial Research*, *8*, 209–218.

Wang, J., DeBoer, J., & DeGroot, K. (2008). Proliferation and differentiation of MC3T3-E1 cells on calcium phosphate/chitosan coatings. *Journal of Dental Research*, *87*, 650–654.

Wang, L. M., & Stegemann, J. P. (2010). Thermogelling chitosan and collagen composite hydrogels initiated with beta-glycerophosphate for bone tissue engineering. *Biomaterials*, *31*, 3976–3985.

Wang, X. L., Wang, X. M., Tan, Y. F., Zhang, B., Gu, Z. W., & Li, X. D. (2009). Synthesis and evaluation of collagen-chitosan-hydroxyapatite nanocomposites for bone grafting. *Journal of Biomedical Materials Research*, *89A*, 1079–1087.

Wilson, O. C., Jr., & Hull, J. R. (2008). Surface modification of nanophase hydroxyapatite with chitosan. *Materials Science and Engineering*, *28C*, 434–437.

Xu, H. H. K., Quinn, J. B., Takagi, S., & Chow, L. C. (2002). Processing and properties of strong and non-rigid calcium phosphate cement. *Journal of Dental Research*, *81*, 219–224.

Xu, Q., Chao, Y. L., & Wan, Q. B. (2009). Health benefit application of functional oligosaccharides. *Carbohydrate Polymers*, *77*, 435–441.

Xua, H. H. K., Smith, D. T., & Simon, C. G. (2004). Strong and bioactive composites containing nano-silica-fused whiskers for bone repair. *Biomaterials*, *25*, 4615–4626.

Yin, Y. J., Zhao, F., Song, X. F., Yao, K. D., Lu, W. W., & Leong, J. C. (2000). Preparation and characterization of hydroxyapatite /chitosan-gelatin network composite. *Journal of Applied Polymer Science*, *77*, 2929–2938.

Yuan, Y., Chesnutt, B. M., Wright, L., Haggard, W. O., & Bumgardner, J. D. (2008). Mechanical property, degradation rate, and bone cell growth of chitosan coated titanium influenced by degree of deacetylation of chitosan. *Journal of Biomedical Materials Research*, *86-B*, 245–252.

Zhao, L. A., Weir, M. D., & Xu, H. H. K. (2010). An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. *Biomaterials*, *31*, 6502–6510.

Zheng, Z. H., Wei, Y. J., Wang, G., Gong, Y. D., & Zhang, X. F. (2009). Surface characterization and cytocompatibility of three chitosan/polycation composite membranes for guided bone regeneration. *Journal of Biomaterials Applications*, *24*, 209–229.

Zhou, H. J., Qian, J. C., Wang, J., Yao, W. T., Liu, C. S., Chen, J. G., et al. (2009). Enhanced bioactivity of bone morphogenetic protein-2 with low dose of 2-N, 6-O-sulfated chitosan in vitro and in vivo. *Biomaterials*, *30*, 1715–1724.

Zou, Q., Li, Y. B., Zhang, L., Zuo, Y., Li, J. F., & Li, J. D. (2009). Antibiotic delivery system using nano-hydroxyapatite/chitosan bone cement consisting of berberine. *Journal of Biomedical Materials Research*, *89A*, 1108–1117.