Natural-Based Nanocomposites for Bone Tissue Engineering and Regenerative Medicine: A Review

Sandra Pina,* Joaquim M. Oliveira, and Rui L. Reis

Tissue engineering and regenerative medicine has been providing exciting technologies for the development of functional substitutes aimed to repair and regenerate damaged tissues and organs. Inspired by the hierarchical nature of bone, nanostructured biomaterials are gaining a singular attention for tissue engineering, owing their ability to promote cell adhesion and proliferation, and hence new bone growth, compared with conventional microsized materials. Of particular interest are nanocomposites involving biopolymeric matrices and bioactive nanosized fillers. Biodegradability, high mechanical strength, and osteointegration and formation of ligamentous tissue are properties required for such materials. Biopolymers are advantageous due to their similarities with extracellular matrices, specific degradation rates, and good biological performance. By its turn, calcium phosphates possess favorable osteoconductivity, resorbability, and biocompatibility. Herein, an overview on the available natural polymer/calcium phosphate nanocomposite materials, their design, and properties is presented. Scaffolds, hydrogels, and fibers as biomimetic strategies for tissue engineering, and processing methodologies are described. The specific biological properties of the nanocomposites, as well as their interaction with cells, including the use of bioactive molecules, are highlighted. Nanocomposites in vivo studies using animal models are also reviewed and discussed.

1. Introduction

Tissue engineering and regenerative medicine (TERM) is a multidisciplinary field of research that employs principles of chemistry, biology, and engineering sciences towards growth, development, and regeneration of damaged tissues or organs.[1] It involves scaffolds combined with cells and suitable biochemical signals, which promote the design of new organs and tissues.

Among all tissues in the body, bone is the most widely investigated for tissue engineering due to its high potential for regeneration. Bone graft materials, as autografts and allografts, have been used to repair bone fractures and other defects, because of their osteoinductive and osteoconductive characteristics.[2] Nevertheless, concern issues are associated with the risk of disease transfer, infection, chronic pain, possible immunogenicity, deficient supply, and increase of operative time and cost.[3]

Despite their scarcity, biomaterials that are able to mimic the structural, mechanical, and biological properties of natural tissues have been attracting a significant attention. Meaningful progress has been made in designing and processing new materials in order to properly address cell activity. This is an important issue to be considered as regeneration processes involve achieving the desired cell function, i.e., stimulate specific cellular responses and activate genes that stimulate cells differentiation and extracellular matrix (ECM) production for enhancing the regeneration of the damaged tissues. Inspired by the nature of bone, three-dimensional, structurally hierarchical constructs and nanocomposites that can comprise several levels of organization, i.e., from the macroscopic tissue arrangement down to the molecular arrangement of proteins have been required.[4] These nanostructured materials can provide enhanced mechanical performance and allow suitable transduction of the mechanical stimuli to the cellular level.[5]

Nanocomposites involving biodegradable and biopolymeric matrices and bioactive/resorbable nanofillers have been considered as a strategy for tissue engineering and regeneration (Figure 1). The fillers with nanosized features can intensely change the physical properties of the polymer matrix, allowing for the engineering of improved biomaterials that the individual materials cannot attain. The nanoparticles have a large surface area when compared to the conventional microsized fillers, which can form a tight interface with the polymeric matrices, offering improved mechanical properties, while maintaining the favourable osteoconductivity and biocompatibility of the fillers, thus influencing protein adsorption, cells adhesion, proliferation and differentiation for new tissue formation.[6]

Biodegradable polymers from natural origin, like polysaccharides (e.g., cellulose, chitin, glycosaminoglycans) and proteins (e.g., collagen, silk, fibrinogen, elastin) hold significant similarities with the ECM, chemical versatility, and good biological performance without toxicity or immunological reactions.[7–9] On the other hand, bioresorbable fillers, such as calcium
phosphates (CaPs) (e.g., β-tricalcium phosphate, hydroxyapatite) have favourable osteoconductivity, resorbability, and biocompatibility. [10]

The present review provides a comprehensive overview of the most recent achievements relating to the design, processing, and properties of resorbable nanocomposites for tissue engineering and regenerative medicine.

2. Natural Polymers and Calcium Phosphate Material Properties

Different materials, such as natural/synthetic polymers, carbon nanotubes, hydroxyapatite (HAp), and silicates have been exploited for nanocomposite designing and processing, attending to diverse needs in TERM. Special interest has been given to the combination of biopolymers (i.e., proteins, polysaccharides, and glycosaminoglycans) and inorganic/ceramic fillers as CaPs, which will provide biomaterial composites with optimized properties. Natural materials are usually the components of the ECM, playing an important role in maintaining their structure. Besides, they have great design advantages and can easily promote cellular adhesion. On the other hand, CaPs are biocompatible, osteoconductive and biodegradable, but they have a limited range of mechanical strength that does not allow load-bearing applications. A brief description of the most promising material properties is herein presented.

2.1. Biopolymers

2.1.1. Proteins

2.1.1.1. Collagen and Gelatin. Collagen and its denatured form, gelatin, are the most preferred ECM proteins used in tissue engineering due to the presence of several functional groups that can enhance osteoblast adhesion and migration. [9] Collagen and gelatin structures are presented in Figure 2.

Collagen is a fibrous protein, the major component of ECM and presents different morphologies in different tissues. It is, for example, found in bone (Type I), cartilage (Type II) and in blood vessel walls (Type III). This protein is non-cytotoxic, biocompatible, and biodegradable, but has low elasticity and mechanical strength, poor dimensional stability due to swelling in vivo, ability of being cross-linked to tailor the mechanical, degradation, and water-uptake properties, and the possibility of an antigenic response. [9] Collagen can be processed in films, [7] fibers, [8,10,12] and foams, [13–15] to engineer various tissues such as bone, cartilage, heart, ligament, and nerve. Collagen is also suitable to produce scaffolds for the culture of mesenchymal stem cells (MSCs) in tissue engineering. [16,17]

Considering the recognized biological properties of collagen, nanocomposites developed from this natural polymer and CaPs show increased mechanical strength as compared to pure collagen. This behavior has been attributed to an increased rigidity of the nanocomposites with the addition of CaPs nanoparticles and to the strong interaction between calcium-binding residues on the polymer macromolecules and the surface of the nanoparticles. [18] Collagen/CaPs nanocomposites have been prepared using different processes, such as the direct addition of nano CaPs to the collagen solution, deposition of CaPs
nanoparticles into collagen, or by electrospinning a mixture of nanosized CaPs and collagen.\[13,19\] In a different approach, the group of Kikuchi prepared a porous HAp/collagen nanocomposite through a titration method for bone tissue engineering, as well as for bone filler.\[20\] Bone marrow cells co-cultured with MG63 osteoblast cells on the nanocomposites showed differentiation to osteoclasts without differentiation supplements.

Gelatin is biocompatible, non-immunogenic and biodegradable.\[9\] Despite gelatin lacks the structural characteristics of collagen, it has higher solubility and lower cost when compared with collagen. Gelatin/CaPs nanocomposites with enhanced mechanical properties and good cell attachment have been developed.\[21–23\] The effects on gelatin-induced microstructures of HAp crystals were investigated by Ching-Chang Ko et al.\[24\] The results showed that increasing the content of gelatin, led to better plane strain modulus and fracture toughness. The gelatin appeared to shorten the HAp crystal distance, which consolidated the internal structure of the composite and made the material more rigid. Azami et al.\[23\] designed a gelatin/HAp nanostructured scaffold with mechanical strength comparable to the spongy bone, as well as, an excellent capacity of cell attachment, migration and penetration into the pores of the nanocomposite.

2.1.1.2. Silk Fibroin. Silks are a class of proteins produced from insects, spiders, and worms, which are composed of fibroin (70–80%), the structural protein of silk fibers, and sericin (20–30%), the water-soluble glue-like protein that encased fibroin.\[26\] Silkworm fibroin is of interest for biomedical engineering and it has to be extracted from the silkworm cocoon by elimination of the sericin via boiling in an alkaline solution. The degummed silkworm silk is then dissolved in lithium bromide, dialyzed and formed in an aqueous silk fibroin (SF) solution. A schematic illustration of the SF solution preparation is shown in

Figure 3. Bombyx mori SF has attracted increasing interest for bone, cartilage, and ligament tissue engineering due to its remarkable properties like elasticity, mechanical strength and toughness, biocompatibility, and biodegradability with controllable degradation rates.\[27,28\] The degradation rates of SF-based scaffolds can be adjusted by changing the crystallinity, molecular weight, porosity, and β-sheet structure. SF is rich in β-sheet structures owing to hydrophobic domains which provide a good resistance to water solubility, and lead to the high mechanical properties of these materials.\[29\] SF can be processed into fibers,\[27,30\] membranes,\[31\] films,\[32\] meshes,\[33\] foams,\[34,35\] and hydrogels\[36\] for the repair/regeneration of several tissues. Silk-based composite scaffolds with enhanced physicochemical and biological properties have been developed for bone tissue engineering.\[29,37,38\] The incorporation of CaPs/silk powders into silk scaffolds showed an improved porous structure, osteogenic differentiation and in vivo bone formation.\[38,39\] Also, the incorporation of HAp in silk foams enhance the osteoconductivity and mechanical properties of the scaffolds.\[35\]

Silk scaffolds in combination with MSCs for bone and ligament tissues engineering have been developed.\[17,40\] He et al.\[40\] demonstrated the fibrocartilaginous differentiation of bone marrow mesenchymal stem cells (BMSCs) in a co-culture system involving BMSCs, fibroblasts, and osteoblasts on a hybrid silk scaffold. The region of the osteoblasts-seeded scaffold was coated with HAp to stimulate bone ingrowth.

2.1.2. Polysaccharides

2.1.2.1. Chitosan. Chitosan is a polysaccharide produced from marine crustacean shells, however, commercially available chitosan is produced from deacetylation of chitin (Figure 4).
which is a natural polysaccharide found in crab, shrimp, lobster, coral, jellyfish, butterfly, ladybug, mushroom and fungi.\textsuperscript{[42,43]} Chitosan is a cationic polymer composed of randomly distributed N-acetyl glucosamine and D-glucosamine, varying in composition, sequence, and molecular chain length. Hence, chitosan enables the electrostatic interaction with negatively charged biomolecules and the interaction with cell membranes. Chitosan has been developed in diverse forms like films,\textsuperscript{[44]} fibers,\textsuperscript{[45]} foams,\textsuperscript{[14,46]} hydrogel,\textsuperscript{[47]} and particles,\textsuperscript{[48]} for applications in bone and cartilage tissue engineering and wound healing, due to the excellent properties like biocompatibility, biodegradability, ability for cell ingrowth, and intrinsic antibacterial nature.\textsuperscript{[49]} However, chitosan itself is not osteoconductive and it has low mechanical strength being unable to support load-bearing. The degradation rate of chitosan is inversely related to the degree of crystallinity and complete degradation can take months.

Hence, a diversity of materials, namely CaPs, has been combined with chitosan to produce stronger scaffolds with enhanced biological properties for bone tissue engineering.\textsuperscript{[50–54]} For example, physicochemical properties and in vitro cytotoxicity of chitosan/CaPs scaffolds were evaluated.\textsuperscript{[50]} It was demonstrated that the scaffolds compressive strength is in the range of trabecular bone, and proliferation and differentiation of MG63 cells on the scaffolds. Similarly, human MSCs cultured on chitosan/HAP composite scaffolds showed increased proliferation when compared to those cultured on pure chitosan.\textsuperscript{[52]} Zhang et al.\textsuperscript{[54]} prepared porous chitosan/
HAp scaffolds with 3D oriented structure showing a high degree of proliferation of MC3T3-E1 cell line, adhesion and alkaline phosphate activity.

2.1.2.2. Alginate. Alginate is a natural polymer that can be obtained from brown algae. It is composed of two monomers, (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) (Figure 5). Alginites extracted from different sources have different M and G contents along with the length of each block, influencing the properties of the alginate. For example, increasing the length of G block and molecular weight, the mechanical properties of the alginate will be improved. Also, high M content are immunogenic and more potent in inducing cytokine production as compared to G content.

Alginate is increasingly investigated for tissue engineering, wound healing and drug delivery, due to its biocompatibility, low toxicity and immunogenicity, and controllable gelation. It is normally used in the form of a hydrogel, but it can also be used as gels and fibers for cell immobilization and proliferation, as well as injectable pastes. Alginate has been combined with inorganic materials to engineer and repair/regeneration bone tissue. Alginate, gelatin, and biphasic CaPs were fabricated by the Schiff-base reaction and demonstrate adjustable gelation and biodegradation time, good mechanical strength, and excellent biocompatibility. Calcium phosphate cement and alginate scaffolds were prepared for the culture and expansion of osteoblastic cells showing an active proliferative potential and osteogenic differentiation. In addition, human umbilical cord MSCs-encapsulating calcium phosphate cement/alginate composite paste have demonstrated mechanical strength matched the reported values of cancellous bone, and the encapsulated cells remained viable and osteo differentiated, yielding high alkaline phosphatase (ALP), osteocalcin, collagen Type I, and osterix gene expressions. Likewise, it was reported that a hybrid scaffold of HAp and alginate hydrogel promote formation of chondrocyte of a calcified cartilage-like matrix in vitro for the regeneration of the osteochondral interface tissue engineering.

2.1.2.3. Gellan Gum and Derivatives. Gellan gum (GG) is an anionic, high molecular weight, extracellular polysaccharide produced by the fermentation of the organism *Sphingomonas elodea* bacterium (originally designated *Pseudomonas elodea*), which lives on the algae *Elodea Canadensis*. GG consists of approximately, 60% glucose, 20% glucuronic acid, and 20% rhamnose as a repeating unit, and two acyl groups, acetate and glycerate bound to glucose residue adjacent to glucuronic acid. It has a high gel strength, excellent stability, and thermally reversible gel, which is formed in the presence of metal ions. GG is available as native form or deacetylated form by removing the acetyl groups, resulting in harder and more brittle gels with higher thermal stability. Also, it is available in a clarified form, by filtration of hot deacetylated GG, for microbiological media, plant tissue culture, and pharmaceutical application purposes.
Methacrylated GG is a derivative form of GG produced through chemical modification by means of methacrylation of low acyl GG.

It has been reported GG to be used in several acellular and cellular tissue engineering strategies. For example, GG-based hydrogels can be tunable for the regeneration of intervertebral disc to improve their mechanical properties, to control endothelial cells infiltration and blood vessel ingrowth. In addition, GG-based hydrogels blended with HAp particles have been proposed for bone and osteochondral applications showing greater mechanical and biological properties in comparison to the polymeric hydrogel. Bilayered GG/GG-HAp hydrogels were produced for osteochondral tissue engineering applications, by joining both solutions of GG 2% (w/v) with and without HAp (20 wt.%) for bony and cartilage parts, respectively. Results showed that the bilayered scaffolds possessed 83.4 ± 0.8% porosity, 279.3 ± 38.6 μm pore size and interconnectivity of 62.2 ± 5.4%.

2.1.3. Glycosaminoglycans

2.1.3.1. Hyaluronic Acid. Hyaluronic acid (HA) is an anionic, non-sulfated glycosaminoglycan consisting of repeating D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine-β-1,4 units. It is one of the major constituent of the ECM present in all connective tissues, presenting good viscoelasticity and water-binding ability, biocompatibility and non-immunogenicity, which make it suitable to be used in several biomedical applications for tissue engineering and regenerative medicine, as well as for cell encapsulation and as a drug delivery system. HA can be chemically modified in order to alter the physico-chemical and biological properties of the resulting materials, and their derivatives and cross-linked materials have been created in different forms such as hydrogels, fibers, meshes, and foams.

HA-based composites aiming to improve structural integrity, fracture strength, and toughness of scaffolds have been reported for tissue engineering applications. In addition, the incorporation of HA in cross-linked networks have shown high potential for the treatment of cartilage damage.

2.2. Calcium Phosphate-Based Materials

2.2.1. Calcium Phosphates. CaPs are the chemical compounds of special interest for human beings due to their similarity with the inorganic part of major normal (bones, teeth and antlers) calcified tissues of mammals. CaPs possess remarkable biocompatibility, osteoconductivity, bioresorbability, and direct bonding to bone. The most relevant CaPs are presented in Table 1. CaPs commonly used for tissue engineering are β-TCP and HAp. β-TCP is a high temperature phase of CaPs, which only can be obtained by its thermal decomposition at temperatures above 800 °C. HAp is highly crystalline and is the most stable and least soluble CaPs in an aqueous solution below pH 4.2. HAp can be prepared using wet methods, such as precipitation, hydrothermal and hydrolysis of other CaPs. HAp can be also obtained from a solid-state reaction of, for example, MCPM, DCPA, DCPD, OCP, with calcium oxide, calcium hydroxide or calcium carbonate, above 1200 °C. The detailed information on HAp synthesis is reported and available elsewhere.

Although β-TCP and HAp have similarities in their chemical composition, they differ in their biological resorbing capability. The resorption of a ceramic HAp is slow, and once implanted
into the body, HAp may remain integrated into the regenerated bone tissue, while \(\beta\)-TCP is completely reabsorbed.\(^{[105]}\) Clinical applications of pure HAp can be improved with the bioreabsorbable \(\beta\)-TCP for better bone regeneration. The main attractive feature of these materials is their ability to form a strong direct bond with the host tissue resulting in a strong interface.\(^{[101]}\)

2.2.2. Ionic Co-Substitutions in Calcium Phosphates

The mineral component of bone is similar to HAp but contains other ions in composition, as illustrated in Table 2.

Table 2. Composition of inorganic phases of adult human calcified tissues.

<table>
<thead>
<tr>
<th>Composition (wt%)</th>
<th>Bone</th>
<th>Enamel</th>
<th>Dentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (Ca)</td>
<td>34.8</td>
<td>36.5</td>
<td>35.1</td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>15.2</td>
<td>17.7</td>
<td>16.9</td>
</tr>
<tr>
<td>Sodium (Na)</td>
<td>0.9</td>
<td>0.50</td>
<td>0.60</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>0.72</td>
<td>0.44</td>
<td>1.23</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>0.03</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>0.0126 – 0.0217</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fluoride (F)</td>
<td>0.03</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Chloride (Cl)</td>
<td>0.13</td>
<td>0.30</td>
<td>0.01</td>
</tr>
<tr>
<td>Carbonates</td>
<td>7.4</td>
<td>3.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Each of the aforementioned elements is considered to play a pivotal role for the behavior of biological apatites leading to better produced biomaterials.\(^{[105]}\) Therefore, the ionic incorporation into the structure of synthetic CaPs phases, namely in \(\beta\)-TCP (Figure 8a) and HAp (Figure 8b), can affect the lattice structure, microstructure, crystallinity, and dissolution rate of CaPs.\(^{[106,107]}\) Magnesium has its own significance in the calcification process and on bone fragility, and has indirect influence on the mineral metabolism;\(^{[108]}\) strontium is considered as a bone-seeking element that presents a beneficial effect on the bone growth, and has the ability to decrease bone resorption and to enhance bone formation;\(^{[109]}\) zinc is able to promote osteoblast cell proliferation and differentiation;\(^{[110]}\) potassium has a versatile nature in the regulation of biochemical process and also an important role in the apatite mineral nucleation process;\(^{[111]}\) sodium has a potential role in cell adhesion and in the bone metabolism and resorption processes;\(^{[112]}\) chlorine has the ability to develop an acidic environment on the surface of bone that activates osteoclasts in the bone resorption process.\(^{[113]}\) In addition, the incorporation of carbonate or fluoride into a DNA–fibronectin–apatite composite layer for tissue engineering aiming to adjust the layer solubility was developed by Yazaki and their co-authors.\(^{[114]}\) The incorporation of carbonate increased the efficiency of gene transfer on the layer, while fluoride decreased the efficiency and delayed the timing of gene transfer dose-dependently. Also, manganese (Mn), detected as minor constituents of teeth and bone, regulates bone remodeling because its low content in the body is associated with the increase of the extracellular concentration of calcium, phosphates and phosphatase.\(^{[115]}\)

Synthesis, structural and mechanical studies, and in vitro analysis of CaPs with ionic incorporation have been reported, namely Mg,\(^{[116]}\) Sr,\(^{[119,120]}\) Zn,\(^{[107,120]}\) Na,\(^{[121]}\) F,\(^{[122]}\) Ag,\(^{[123,124]}\) Mn,\(^{[127]}\) Si,\(^[120,126]\) and Ba.\(^{[127]}\) Kose et al.\(^{[123]}\) showed that silver ion-containing CaP-based ceramic nanopowder-coated implants led to an increase in resistance to bacterial colonization compared to HAp-coated, and uncoated titanium implants. Torres et al.\(^{[125]}\) prepared Mn-doped \(\beta\)-TCP powders and reported that Mn-doping significantly affected the structure and morphology of \(\beta\)-TCP powders. In vitro proliferation and differentiation assays of MC3T3-E1 osteoblast-like cells, grown in the presence of the powders, revealed that the biological benefits of...
Mn-doped \( \beta \)-TCP are limited to lower Mn incorporation levels and potentially related to their surface microstructure. Another interesting work prepared Si-substituted nano-CaPs followed by the fabrication of multilayered scaffolds.\(^{126}\) In that study, it was reported that the silicon content facilitates a progressive change of nano-HAp structure to nano-TCP, and enhanced adhesion, spreading, growth and proliferation of osteoblasts on the scaffolds, which might be considered for bone tissue engineering, with potential applications in bone reconstruction and regeneration.

2.2.3. **Calcium Phosphate Cements**

Calcium phosphate-based cements (CPC) as injectable pastes with options for cell delivery in tissue engineering of bone are also a strong subject of research.\(^{128,129}\) CPC are made of an aqueous solution and CaPs, which upon mixing, dissolve and precipitate into a less soluble CaP and set by the entanglement of the growth crystals, providing a mechanical rigidity to the cement.\(^{130}\) When the paste becomes sufficiently stiff, it can be injected into a defect as a substitute for the damaged part of bone, where it hardens in situ within the operating theatre. The handling characteristics and the ability to harden at body temperature make it an attractive delivery vehicle for therapeutic agents in orthopaedic applications. For example, modified CPC with alginate gel showed good cell proliferation, and increased osteogenic analysis associated with hMSCs, suggesting that this material can be used as a cell delivery vehicle for bone regeneration.\(^{128}\) In addition, CPC loaded with recombinant human bone morphogenetic protein (rh-BMP-2) revealed significantly accelerated healing in bone defects.\(^{131}\) Czechowska et al.\(^{132}\) prepared a cement-type implant material composed of \( \alpha \)-TCP and chitosan with the chemical stability and high bioactive potential demonstrated through in vitro studies in simulated body fluid.

3. **Biomimetic Nanocomposites Based on Biopolymers and Calcium Phosphates**

Biomimetic strategies to develop nanocomposites for TERM rely on bioactive structures with controlled geometry able to replicate the natural ECM found in vivo and their ability to direct cell-matrix and cell–cell interactions.

Nanocomposites consist of a polymer matrix combined with nanosized CaPs, which allows tailoring the desired degradation and resorption kinetics of the matrix. Furthermore, nanosized CaPs are able to improve the tissue bonding behavior of the polymeric materials, as well as, cell adhesion and differentiation. Among the biomimetic nanocomposites that have been developed to engineer different tissues, three-dimensional (3D) porous scaffolds, hydrogels, and nanofibrous scaffolds with controlled geometry and structures are the ones selected for discussion due to its important role in tissue engineering scaffolding. Each type of material offers its own advantages and disadvantages in mimicking the organization of native tissue structure. A description of each design is issued as follows.

3.1. **Nanocomposite Porous Scaffolds**

Scaffolds are designed to act as a 3D support structure to the surrounding bone tissue mimicking ECM, with advantageous characteristics, including: (i) porous structure that promotes cell-biomaterial interactions, cell adhesion, growth and migration, (ii) interconnected pores to facilitate transport of mass, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation, (iii) adequate mechanical properties as tensile strength and elasticity, (iv) controlled degradation, (v) synthesis of new bone formation with homogeneous distribution to avoid necrosis, and (vi) minimal degree of inflammation or toxicity in vivo.\(^{133}\) Furthermore, scaffolds have desirable characteristics for cell transfer into a defect site and to restrict cell loss, instead of simple injection of cells to the defects.\(^{134}\)
Composite scaffolds commercially available are manufactured from collagen and CaPs consisting of HAp, β-TCP, and biphasic CaP that combine both structures to tune the degradability. Collagen is the most abundant polymer in bone tissue, thus is one of the most obvious candidates for combination with nanosized CaPs.

Nanocomposite scaffolds have been fabricated with specific pore size, porosity, surface-area-to-volume ratio and crystallinity, by means of different technologies including foam replica method, solvent casting and particulate-leaching, freeze-drying, phase separation, gas foaming, rapid prototyping, and electrospinning. The challenge is the production of scaffolds ensuring a good compatibility between the phases while keeping the porous structure and the mechanical properties. Also, it is important to achieve a homogeneous distribution of nanosized CaP particles inside the polymer. Methods used to maximize the CaP nanoparticles distribution include precipitation in situ of the mineral crystals in the polymer matrix or by the use of dispersants as sodium citrate. For example, nanocomposite scaffolds made of SF and CaPs by using an in situ synthesis method, where phosphate ions are added into the calcium chloride solution with dissolved SF, followed by the addition of ammonia dibasic phosphate solution by means of salt-leaching/freezedrying techniques. Figure 9 presents scanning electron microscopy (SEM) images of the scaffolds showing a macro/micro porous structure. The scaffolds presented highly interconnected macro-pores with sizes around 500 µm, and micro-pores with a size range of 10–100 µm, as shown in Figure 9a,b,c. It is also shown the formation of cauliflower-like apatite clusters with a size of 700 nm, on the surface of the scaffold (Figure 9d). The size of the CaP particles in the composite scaffolds was less than 200 nm confirmed through energy dispersive X-ray detector spectra (Figure 9f).

Kim et al. prepared aqueous-derived SF scaffolds with the addition of polyaspartic acid, followed by the controlled deposition of CaPs by exposure to chloride and sodium phosphate monobasic solutions. It was observed that the carboxyl groups from the polyaspartic acid enhanced apatite deposition on the SF substrates. In another study, HAp was synthesized in SF by the addition of phosphate ions into the calcium chloride/ethanol/water solution with dissolved SF. On the other hand, Bhumiratana and his colleagues embedded HAp micro-particles in silk foams to generate highly osteogenic composite scaffolds capable of inducing the formation of tissue-engineered bone. Chen et al. developed collagen-SF/HAp nanocomposites via an in situ precipitation technique. Morphology studies showed that HAp particles were distributed uniformly in the polymer matrix and possess a size ranging from 30 to 100 nm, which were composed of more fine sub-particles with sizes ranging from of 2–5 nm without regular crystallographic orientation. All these strategies allowed the formation of nanosized CaP particles inside the polymeric matrix.

The design, characterization, and biological evaluation of various natural polymer/CaP nanocomposite scaffolds with the potential for tissue engineering and regenerative medicine have been reported. Cunniffe’s group developed a collagen/nano-HAp composite scaffold with higher mechanical properties and the same high biological activity compared with the collagen control scaffold (5.50 ± 1.70 vs 0.30 ± 0.09 kPa). Yan et al. prepared a composite scaffold made from SF and nanosized CaP with self-mineralization...
capability and no cytotoxicity. Barbani et al.\textsuperscript{[21]} produced gelatin/ HAp nanocomposite scaffolds, using a freeze-drying technique, with elastic modulus similar to natural bone. Biological tests showed good adhesion and proliferation of human MSCs. In another study, a bio-hybrid SF/CaP/poly lactic-co-glycolic acid (PLGA) nanocomposite scaffold was produced using freeze-drying and electrospinning to be used as a delivery system.\textsuperscript{[151]} It was shown that these nanocomposites provide an optimal microenvironment in terms of porosity, physical and chemical structure. The SF/CaP microstructure has shown highly porous structure suitable for the transportation of nutrients and oxygen. In addition, PLGA was successfully electrospun on the surface of VEGF loaded SF/CaP nanocomposite and formed highly uniform nanofibers with a diameter of 300–500 nm. The porosity of freeze-dried substrates was in the range of 70–75%, which considered enhancing the exchange of nutrients and waste materials. Eftekahi et al.\textsuperscript{[152]} developed a novel porous nanocomposite composed of cotton-sourced cellulose microcrystals, HAp nanoparticles and poly l-lactide acid with a pretreatment of particles by using sodium dodecyl sulfate (SDS) as a coupling agent. The results indicated that the incorporation of cellulose and HAp nanoparticles improves the mechanical strength of the nanocomposites to be used for bone tissue regeneration. Another work was reported collagen/nano-HAp biocomposite scaffolds development for bone regeneration. Moreover, hydrogel porosity may be controlled within a nanocomposite hydrogel. Arrow indicates the polymer-nanoparticle aggregates. \textsuperscript{[160]} The composites showed improved mechanical properties and high cellular proliferation.

3.2. Nanocomposite Hydrogels

Hydrogels consist of a 3D network that is highly hydrated, but mechanically fragile due to their randomly arranged chemically cross-linked network.\textsuperscript{[156]} Hydrogel materials hold structural and compositional similarities with the ECM making them attractive scaffolds owing to their swollen network structure, biocompatibility, efficient mass transfer, and ability to encapsulate cells and biomolecules.\textsuperscript{[154]} These properties are influenced by the nature of the polymer chains and degree of cross-linking, molecular arrangement, and the amount of water they absorb.\textsuperscript{[155]} Hydrogels usually exhibit a hydrophilic network porous structure with interconnected pores (>10 µm) to allow cell infiltration and deployment, and provide an increased surface area for cell attachment and interaction. Moreover, hydrogel porosity may be controlled by solvent casting/particulate leaching, phase separation, gas foaming, solvent evaporation, freeze-drying, and blending with non-cross-linkable linear polymers.\textsuperscript{[156]} Hydrogel networks can be engineered, into different sizes and shapes, as thin films, sheets, spheres, rods, hollow tubes, and bellows, due to their unique physical properties.\textsuperscript{[157]} From the tissue engineering point of view, hydrogels can be used as 3D structures that organize cells and present stimuli to direct the formation of a desired tissue, as space filling agents, and as delivery vehicles for bioactive molecules. \textsuperscript{[158]} Excellent reviews regarding a depth description of hydrogel properties are well published.\textsuperscript{[154,159]}

Nanocomposite hydrogels are defined as an organic-inorganic network structure, chemically or physically cross-linked with nanoparticles.\textsuperscript{[160]} Compared to the conventional ones, nanocomposite hydrogels have enhanced chemical, physical, electrical and biological properties, mainly attributed to the improved interactions between the polymeric network and the nanoparticles. Nevertheless, hydrogel nanocomposites lack of some essential features, as biodegradation and stimuli responsiveness, which can be enhanced by combining multiple phases within a nanocomposite hydrogel.\textsuperscript{[161]}

Hydrogels produced from inorganic nanoparticles, as nanosized CaPs, incorporating a natural or synthetic polymeric hydrogel matrix can provide not only greater mechanical strength, but also tuning the bioactive characteristics to the network. For example, an injectable and thermo-sensitive poly(ethylene glycol) (PEG) – poly(ε-caprolactone) (PCL) – PEG copolymer/collagen/nano-HAp hydrogel composite for guided bone regeneration was developed and investigated in vivo biocompatibility and biodegradability by implanting the hydrogel composite in rats by Fu et al.\textsuperscript{[162]} The results showed good biocompatibility, biodegradability, and new bone tissue formation of the nanocomposite. In another study, nanosized HAp was incorporated into a PEG matrix aiming the production of highly tough and elastomeric nanocomposite hydrogels.\textsuperscript{[163]} The morphologies of the nanocomposite hydrogel fractured surfaces show highly porous structures and interconnected porous structures with pore sizes between 100–300 nm (Figure 10). The incorporation of HAp nanoparticles enhanced the mechanical properties of the nanocomposite networks, due to the nanoparticle interactions that interfere with the cross-linking of PEG during the photopolymerization. Additionally, the presence of nano-HAp provided osteoblast cell adhesion and bioactive attachment sites to the osteoblast cells, when compared with PEG hydrogels.

Figure 10. Microstructure of the nanocomposite hydrogel with 5% of nanosized HAp concentration. Arrow indicates the polymer-nanoparticle aggregates. Scale bar 2 µm. Adapted with permission.\textsuperscript{[163]} Copyright 2011, American Chemical Society.
To date, scarce reports on nanocomposite hydrogels produced from natural polymers and nanosized CaP can be found.\textsuperscript{[162,164,165]} Nejadnik et al.\textsuperscript{[164]} developed an injectable, cohesive nanocomposite hydrogel based on CaP nanoparticles and bisphosphonate-functionalized HA. The results showed that the nanocomposites display a capacity for self-healing and adhesiveness to mineral surfaces, such as enamel and HAp. Moreover, the nanocomposites are biodegradable upon in vitro and in vivo testing and show bone interactive capacity evidenced by bone ingrowth into material remnants. Another work describes the creation of nanocomposites hydrogel made of xanthan cross-linked with citric acid and HAp hydrogel, by using xanthan modified nano-HAp and its equivalent Sr substituted.\textsuperscript{[165]} The enrichment of HAp nanoparticles by xanthan enhanced the colloidal stability and compatibility between the chains and the nanoparticles. Nanocomposites presented improved mechanical properties in comparison to bare xanthan networks or to nanocomposites with HAp. Also, the ALP activity increased in both nanocomposites, being more pronounced for the samples substituted with Sr.

3.3. Nanocomposite Fibrous Scaffolds

Fibrous scaffolds are a good option to mimic the fibrous structure of the native ECM, with advantageous morphologies to porous scaffolds and hydrogels.\textsuperscript{[166]} Nanofibers display a similarity to the network of collagen fibrils each about 50–500 nm diameter. They possess high porosities (up to 95%), isotropic structures, and homogeneous fiber size and pore distribution. In addition, this type of scaffold is able for cell adhesion and proliferation, since cells adhere and organize well around fibers with dimensions smaller than the diameter of the cells.\textsuperscript{[167]} The mechanical properties of fibrous scaffolds are dependent on their composition, fiber diameter and orientation.\textsuperscript{[168]} Furthermore, nanoscale fibrous scaffolds with well-controlled patterned structures have received particular interest to enhance cell functions as cell adhesion, migration, proliferation and differentiation.\textsuperscript{[169]} A nanofiber scaffold has been used in tissue engineering for bone, cartilage, ligament, skeletal muscle, skin, neural tissue engineering, and as vehicle for the controlled delivery of drugs, proteins, and DNA.\textsuperscript{[170]}

Nanocomposite fibrous scaffolds can be obtained from a polymer and a nanosized ceramic phase, by using molecular self-assembly, phase separation, and electrospinning fabrication techniques. Molecular self-assembly is able to produce highly ordered nanofibers but it is limited to molecules for self-assembly. A phase separation method can only produce randomly distributed fibers in the sub-micrometer range and allows for the control of pore architectures. Electrospinning can generate fibers, from nano- to micro-size, with controllable pore size, fiber size and stiffness, and matrix turnover, being the most widely studied technique.\textsuperscript{[171]} Also, the incorporation of bioactive agents to electrospun fibers can lead to enhanced biomimetic scaffolds, since cell-substrate interaction is strongly affected by the presence of chemical cues, able to support cell adhesion, proliferation and differentiation.\textsuperscript{[172]}

Electrospun nanofiber-based natural polymer and CaP composites are being explored as scaffolds similar to natural ECM for TERM applications.\textsuperscript{[53,142,173–176]} Chae et al.\textsuperscript{[142]} successfully fabricated HAp/alginate nanocomposite fibrous scaffolds via electrospinning and a novel in situ synthesis of HAp that mimics mineralized collagen fibrils in bone tissue. They hypothesized that the in situ nucleation and crystal growth of HAp on electrospun nanofibers during the cross-linking treatment would induce homogeneous deposition of the HAp nanocrystals on the nanofibers and overcome the drawbacks of the mechanically blended/electrospun composite nanofibers. Results showed that the electrospun HAp/alginate scaffold composed of random nanofibers containing homogeneously distributed HAp nanocrystals presented in Figure 11. The in vitro cell study showed good adhesion of rat calvarial

Figure 11. Microscopy images of a) electrospun HAp/alginate and b) cross-linked/in situ synthesized HAp/alginate scaffolds. c) Illustration of the cross-linked/in situ synthesized HAp/alginate scaffold. Adapted with permission.\textsuperscript{[142]} Copyright 2013, Springer.
osteoblasts to the scaffolds, being more stable attached on HAp/alginate scaffolds than attachment on pure alginate, presenting a stretched and elongated shape into a spindle-shape on the HAp/alginate scaffolds. Liu et al.\textsuperscript{[173]} developed a nanofibrous gelatin/apatite composite scaffold using thermally induced phase separation technique for bone tissue engineering. The scaffolds showed high porosity and interconnectivity. Also, the composite scaffolds demonstrated high mechanical strength and favorable osteoblastic cell differentiation due to the addition of apatite. Kim et al.\textsuperscript{[175]} produced electrospun SF composite scaffolds by uniformly dispersing HAp nanoparticles within SF nanofibers. The composite scaffolds showed increased mechanical properties with the increase of HAp content up to 20 wt% and good biocompatibility.

A different approach was used to fabricate SF nanofibers containing HAp nanoparticles with desirable properties.\textsuperscript{[176]} This technique employing a three-way stopcock connector was used to electrospun a blend solution of SF and HAp together in aqueous solution. In Figure 12, SF nanofibers and SF nanofibers modified with HAp nanoparticles are demonstrated. The authors concluded that HAp nanoparticles enhanced the $\beta$-sheet conformation of SF improving their properties. Also, the nanofibers showed non-toxic behavior and good attachment of fibroblast cells after incubation.

HAp nanoparticles have also been used in combination with chitosan to produce genipin-crosslinked nanofibrous scaffolds.\textsuperscript{[177]} These nanofibrous composites were able not only to support the adhesion and proliferation of mouse osteoblast-like cells but also to induce their osteogenic differentiation. This was evidenced by increased levels of expression and activity of the early osteogenic marker ALP in cells cultured on the composite material in comparison to those grown in chitosan scaffolds.

4. Processing Methodology in Nanocomposites Engineering

Biopolymer/CaP nanocomposites are often obtained from CaP nanopowders/nanoparticles, which have been made through a large number of methods, namely wet chemical precipitation, sol-gel synthesis, hydrothermal synthesis, mechanochemical synthesis, microwave processing, and spray-drying methods.

Conventional techniques have commonly been used for scaffolding fabrication such as, foam replica method, solvent casting and particulate-leaching, freeze-drying, gas foaming and phase separation. These conventional methods are often inexpensive, simple to design, and flexible to optimize or modulate physico-chemical properties.

Rapid prototyping and electrospinning for the production of 3D structures and nanofibers, respectively, are sophisticated techniques, which have attracted a great deal of attention due to their ability to mimic new tissue structures and the possibility of incorporating pharmaceutical agents. Molecular self-assembly is another strategy available for the production of nanofibers.

The aforementioned processing methodologies in nanocomposite engineering are overviewed as follows.

4.1. Synthesis of CaP Nanopowders

CaP nanopowders/nanoparticles have been produced using a large number of methods, such as wet chemical precipitation,\textsuperscript{[178-180]} sol-gel synthesis,\textsuperscript{[178,181,182]} hydrothermal synthesis,\textsuperscript{[182,183]} mechanochemical synthesis,\textsuperscript{[184]} microwave
irradiation-assisted processing, and the spray-drying method. Among them, the wet chemical process, also known as aqueous precipitation, is the most extensively investigated technique for CaP nanoparticle synthesis, followed by heat treatment according to the desired CaPs structure. The advantage of this method relies on the homogeneity of the final product, and the easiness of controlling parameters such as the precipitation temperature, pH, and the presence of additives during the synthesis, which can affect the shape, stoichiometry, dimensions and specific surface area of the nanoparticles, and thereupon, their biodegradation properties. The aqueous precipitation method often involves the reaction between calcium nitrate and diammonium hydrogen phosphate solutions as the chemical precursors for Ca and P, respectively. The addition of chemical agents such as citric acid, sodium citrate, amino acids and ethylene diamine tetra acetic acid (EDTA) have been used to stabilize the structure of the CaP nanoparticles. CaP particles should be uniform in size and morphology, and spherical, for a satisfactory bioresorbability. Therefore, precipitation procedures, yielding a high density of crystallization nuclei, should be implemented in order to obtain nanosized particles.

4.2. Foam Replica Method

Foam replica method is based in the impregnation of an aqueous suspension in porous synthetic polymeric (usually polyurethane) foams until the total filling of the pores. The impregnated foam is then passed through rollers or centrifuged to remove the excess suspension and allow the formation of a thin coating over the original structure and left to dry. Then, the foams are carefully heated at temperatures between 300–800 °C, usually at heating rates lower than 1 °C/min, for the slow decomposition and diffusion of the polymeric template, and thus a porous structure is obtained. Finally, the scaffolds are densified by sintering at temperatures ranging from 1100 °C to 1700 °C, depending on the material.

This method allows the production of macroporous structures with a reticulated structure of highly interconnected pore sizes ranging from 200 µm to 3 mm at total open porosity levels between 40 and 95% (Figure 13). However, the mechanical strength of the produced structures can be degraded by the formation of cracked struts during the decomposition of the foam (Figure 13b).

4.3. Solvent Casting and Particulate-Leaching

A combination of solvent casting and particulate-leaching methodologies is widely used to successfully fabricate 3D porous scaffolds. This is a process based on the dispersion of a salt (e.g., sodium chloride, ammonium bicarbonate, and glucose) in a polymer dissolved in an organic solvent. The solvent is eliminated, resulting in the solidification of the polymer. Then, the salt crystals are leached away using water to form the pores of the scaffolds (Figure 14). The pore size can be controlled by the size of the salt crystals and the porosity by the salt/polymer ratio. Porosity values of up to 93% and average pore diameters of about 300 µm can be formed using this process. However, certain critical variables such as pore shape and interconnectivity of the pores are not controlled with this method.

4.4. Freeze-Drying

Freeze-drying, also known as lyophilization, is a dehydration technique, which allows freezing a material followed by drying...
under vacuum. Firstly, a polymer is dissolved into a solvent with the addition of water, followed by freezing at different temperatures and rates, forming ice crystals and forcing the molecules to aggregate in the interstitial spaces. Then, the frozen material is dried at a low temperature under reduced pressure to remove the dispersed water and the solvent, thus leaving a porous polymeric structure. One of the main advantages of this process is the possibility to adjust the porosity of the foams to the desired needs by manipulating the freezing time and annealing stage. However, scaffolds produced using this method are not suitable for the use in conditions that imply mechanical stress, which is a consequence of the difficulty in maintaining proper structural stability and mechanical properties after hydration. Moreover, the pore size is relatively small and the porosity is often irregular.

Silk-based scaffolds using salt-leaching and freeze-drying suitable for meniscus and cartilage tissue engineering applications has been prepared (Figure 15). Morphological analysis showed that the structures possessed both macro and micro porosity with values up to 91% and the mean interconnectivity up to 97%, depending on the polymer concentration in the structures.

4.5. Gas Foaming

The gas foaming technique utilizes high pressure CO$_2$ gas dispersed throughout a polymer mixed with a porogen (e.g., sodium chloride), until saturation. The solubility of CO$_2$ is decreased rapidly until to atmospheric level resulting in nucleation and growth of gas bubbles. After completion of the foaming process, the porogen is removed and a highly interconnected pore structure is created. The morphology of the scaffolds depends on the processing conditions, namely pressure, temperature, and venting time (Figure 16). This technique does not require the use of organic solvents and high temperature as for the most fabrication techniques.

Porous polymeric foams prepared using the gas foaming technique has emerged in recent years with application as scaffolds for bone tissue engineering. PCL/HAp nanocomposite scaffolds showed an overall porosity ranging from 75.7% to 83.4%, microporosity in the 20—100 µm range and macroporosity in the range 200—600 µm. Results indicated that the micro-architecture of the pore structure of the scaffolds plays a crucial role in defining the cell seeding efficiency, proliferation, and osteogenic differentiation.

4.6. Phase Separation

The phase separation technique is based on the separation into more than one phase in order to lower the system with free energy. Briefly, a polymer solution separates into two phases, a polymer-rich phase and a polymer-lean phase, and when the solvent is removed, the polymer-rich phase solidifies. This technique has been used to fabricate porous membranes for filtration and separation but has the disadvantage of forming pores with diameters on the order of a few to tens of
micrometers that are not uniformly distributed. However, controlled phase separation, as thermally induced phase separation, has been used for scaffold fabrication. This process is based on the decrease in solubility associated with a temperature increase. After demixing is induced, the solvent can be removed by using extraction, evaporation, and freeze-drying methods. Highly porous scaffolds made of PLLA by thermally induced phase separation starting from 1,4-dioxane/PLLA solutions have been prepared for applications in vascular nets and angiogenesis (Figure 17). Microscopy images displayed pores organized into domains with different orientations, showing dendrite-like and spherical structures. Overall and interconnected porosity values were in the range of 77–93% and 68–91%, respectively, depending on the phase separation temperature.

4.7. Rapid Prototyping

Rapid prototyping (RP), also known as solid freeform fabrication or additive manufacturing, is an advanced technique that uses computer generated data, such as Computer Aided Design (CAD), Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) data, to design complex-shaped objects. The fabrication process involves a 3D design of the scaffold, which is directly produced layer-by-layer. As a result, this technique allows a precise control of the pore size, geometry, and interconnectivity, which enables tuning cells infiltration and behavior into the scaffold.

Robocasting is a specific type of RP that allows the fabrication of a 3D structure by using a robotic arm to force the extrusion of a colloidal suspension through a microsized nozzle (Figure 18a). The ceramic ink placed in the injection syringe is then forced through the nozzle into an oil bath, resulting in porous scaffolds with a controlled architecture, enhanced compressive strength and toughness as has been demonstrated with a porous \( \beta \)-TCP scaffold (Figure 18b-d).

4.8. Electrospinning

Electrospinning has received considerable interest for the use in tissue engineering aimed at producing polymeric nanofibers non-woven membranes scaffolds to the order of nanometers with large surface areas and superior mechanical properties. This process is controlled by a high electric field applied between two electrodes, being one placed in the polymer solution and the other is placed in the collector. During the electrospinning process, a polymer solution is held at a needle tip by surface tension. This electrostatic force opposes the surface tension, causing the initiation of a jet, producing the fibers. As the jet travels, the solvent evaporates and the nanofibers are deposited in the collector (Figure 19A). The characteristics of the nanofibers depend on various properties of the solution and on the processing parameters. This process enables the production of scaffolds with interconnected pore structure and sub-micrometer diameter fibers in a simpler and more cost-effective way than other techniques like phase separation.

Composite branched-star PCL (\( \beta \)-PCL) fiber meshes loaded with HAp nanoparticles and clodronate produced by electrospinning have been developed for bone repair and tissue engineering. Results showed that the fibrous structure is composed of randomly oriented ultra-fine fibers and that the inclusion of the active agents into polymer fibers influenced the mesh morphology (Figure 19B).

4.9. Molecular Self-Assembly

Molecular self-assembly is a useful approach for producing supramolecular architectures that relies in the potential of molecules to spontaneously re-arrange themselves into well organized and stable structures under thermodynamic equilibrium conditions. Such molecular interactions are of non-covalent nature and can occur through hydrogen and ionic bonds, hydrophobic and van der Waals forces, metal coordination, and
Figure 18. Schematic of robocasting fabrication process (a) and obtained microstructures of β-TCP scaffolds: b) general view, c) XY plane view and d) β-TCP rods. Adapted with permission.[204] Copyright 2006, Elsevier.

Figure 19. A) Schematic of electrospinning process. B) Microscopy images of electrospun scaffolds of the composites: a) PCL/nHAp, b) PCL/clodronate, and c) PCL/nHAp/clodronate. Adapted with permission.[205] Copyright 2011, MDPI. Adapted with permission.[207] Copyright 2010, John Wiley and Sons.
electromagnetic interactions.\cite{208} Self-assembly can occur spontaneously in nature, as the self-assembly of the lipid bilayer membrane in cells. It usually results in an increase in internal organization of the system, thus being referred as a “bottom-up” manufacturing technique. Molecular self-assembly is a strategy for developing nanofibrous materials with the potential for tissue engineering (Figure 20).\cite{209,210} On a molecular scale, the accurate and controlled application of intermolecular forces can lead to new and previously unachievable nanostructures. Thus, the mechanical properties and the release profiles of the assembled materials can be tailored specifically for their intended use by appropriate design.\cite{211}

5. Natural Polymer/Calcium Phosphate Nanocomposites – Cell Interactions

Implants to be used for tissue engineering and regenerative medicine have been designed and characterized based on principles used in some forms of in vitro biomineralization, such as immersion in a simulated body fluid (SBF) or using cells (e.g. osteoblasts or stem cells) in static cultures or in bioreactor systems under dynamic conditions.

The use of regulatory signals, including biochemical and/or biophysical stimuli in order to direct gene expression and cellular responses for inducing new tissue formation has also been exploited. In addition, in vivo performance using animal models have been used to evaluate the functionality and biocompatibility of the biomaterials.

5.1. In Vitro Biomineralization Approaches

For over two decades, in vitro mineralization assays have been used as a biomimetic route to evaluate the potential bioactivity of the biomaterials in vivo. The common in vitro studies are performed by immersing the materials in SBF, as proposed by Kokubo, which serves as a source of Ca$^{2+}$ and PO$_4^{3-}$, or by static cultures cell seeding.\cite{212} However, these in vitro culture techniques have nutrient and mass transfer limitations that must be overcome to increase the feasibility of cell-based tissue engineering strategies.

A different in vitro biomimetic approach including dynamic studies using bioreactor systems has been used to overcome the aforementioned limitations by continuously mixing media and by convectively transporting nutrients to cells, with appropriate mechanical stresses.\cite{213} A bioreactor is a culture system designed to support or expand a population of cells through dynamic culture and a controlled environment. Besides, when considering 3D porous architectures, dynamic mineralization environments can also be suitable to promote a homogeneous formation of an apatite layer inside the structure. Figure 21 displays the cell distribution in the scaffolds after cell seeding in static and non-static conditions. Static culture seeding promotes cell attachment and proliferation on the outer edge of the scaffolds, while non-static culture strategies encourage homogeneous cell seeding and proliferation of the scaffolds, as it enhances nutrient diffusion.

A wide array of bioreactor systems for bone tissue engineering has been developed, as spinner flasks,\cite{215} rotating wall vessels,\cite{216} and perfusion systems.\cite{217} Spinner flask and rotating wall systems create a homogenous media on the exterior of the scaffolds, while on perfusion systems, the media is perfused through the pores of the scaffolds, which enables local supply of nutrients providing a better control of the cell microenvironment.\cite{218}

Some authors have studied the mineralization of apatite layers under dynamic conditions using bioreactor systems.\cite{213,219} Oliveira et al.\cite{220} showed that bone-like apatite layers have been formed on the surface and inside of starch/PCL scaffolds. Also, this process was accelerated under flow perfusion conditions, when compared with static and agitated conditions.

Perfusion bioreactor systems have been also reported to improve human mesenchymal stem cells (hMSCs) proliferation and osteogenesis for tissue engineering.\cite{221,222} For example, hMSCs cultured on nanofibrous electrospun PLGA/PCL scaffolds to be implanted into rat femoral condyle defects, showed a superior bone regeneration in groups where implants were cultured in a with bioreactor as compared to that scaffolds cultured in static conditions.\cite{221} Also, Canadas et al.\cite{223} developed low acyl GG (LAGG) – LAGG/HA bilayered structures integrating cartilage- and bone-like layers, respectively, to allow the co-culture of rabbit adipose stem cells (rASCs)-derived osteoblast and chondrocytes. The use of a successfully fabricated rotational dual chamber bioreactor enabled a higher diffusion of medium into the structure, while allowing the use of two different culture mediums for each layer. This device also ensured a homogeneous cell distribution throughout the scaffold, as well as the introduction of mechanical stimuli such as 180° stirring and compression of the top layer. Another study investigated the attachment of cardiac stem cells (CSCs)
to composites of collagen/poly(glycolic acid) nanofibers using culture in a bioreactor perfusion system. It was shown that the attachment and proliferation of CSCs in the 3D culture was enhanced by incubation in a bioreactor perfusion system compared with static culture systems.

5.2. Encapsulation and Release of Bioactive Molecules and Cells

5.2.1. Bioactive Molecules

One of the significant challenges in TERM is to fabricate scaffolds associated with bioactive signaling molecules that provide important cues and signals that can promote cell adhesion, proliferation, differentiation, and metabolic activity for the in vivo regeneration process. These molecules are grouped in mitogens (that stimulate cell division), growth factors (with proliferation-inducing effects), and morphogens (that control generation of tissue form). The most common bioactive molecules applied in TERM are provided in Table 3.

![Figure 21. Static and dynamic cell attachment and proliferation onto the scaffolds. Reprinted with permission.](image)

Table 3. Bioactive molecules for tissue engineering and regenerative medicine applications.

<table>
<thead>
<tr>
<th>Bioactive molecules</th>
<th>Tissues</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDFG (platelet-derived growth factor)</td>
<td>Bone, cartilage, skin, muscle, blood vessel</td>
<td>Mitogen for mesenchymal cells. Regulation of growth and division of cells</td>
<td>[227]</td>
</tr>
<tr>
<td>FGF (fibroblast growth factor)</td>
<td>Bone, skin, nerve, blood vessel</td>
<td>Migration, proliferation and survival of endothelial cells</td>
<td>[228,229]</td>
</tr>
<tr>
<td>IGF (insulin-like growth factor)</td>
<td>Bone, cartilage, liver, lung, kidney, skin, nerve</td>
<td>Cell proliferation, inhibition of cell apoptosis, and bone matrix formation</td>
<td>[230]</td>
</tr>
<tr>
<td>TGF (transforming growth factor)</td>
<td>Bone, cartilage</td>
<td>Wound healing and increase cell proliferation and differentiation</td>
<td>[228,231]</td>
</tr>
<tr>
<td>BMPs (bone morphogenetic protein)</td>
<td>Bone, cartilage</td>
<td>Osteoblast cells differentiation and migration</td>
<td>[226]</td>
</tr>
<tr>
<td>EGF (epidermal growth factor)</td>
<td>Skin, nerve</td>
<td>Cell growth, proliferation and differentiation</td>
<td>[232]</td>
</tr>
<tr>
<td>VEGF (vascular endothelial growth factor)</td>
<td>Blood vessel</td>
<td>Migration, proliferation and survival of endothelial cells.</td>
<td>[233]</td>
</tr>
</tbody>
</table>
followed by the BMP-2 and HAp groups within 4 weeks of implantation. Nevertheless, other biomolecules have been encapsulated in CaP-based nanocomposites with specific functions that can be used as part of tissue engineering therapies. Farokhi et al. developed a bio-hybrid SF/CaP/PLGA nanocomposite scaffold to be used as VEGF delivery system. It was shown that the release profile of VEGF during 28 days has established the efficacy of the scaffold as a sustained delivery system. In fact, the bioactivity of the released VEGF was maintained about 83%. Besides, the scaffolds exhibited good biocompatibility, osteoblast cell adhesion, proliferation and alkaline phosphatase activity. The histology analysis has shown new bone formation after 10 weeks of implantation in a rabbit animal model. Keeney et al. evaluated the capability of a collagen/CaP scaffold to deliver naked plasmid DNA and mediate transfection in vivo, as well as, a plasmid encoding pVEGF165 to promote angiogenesis, and bone formation, in a mouse intra-femoral model. Results showed that the scaffolds with plasmids had higher bone volume in mouse defects in comparison to scaffolds alone. A different delivery system using anionic nanoparticles for small interfering RNA (siRNA) delivery for anticancer therapy was reported. PEG – Carboxymethylchitosan/CaP hybrid anionic nanoparticles showed delivering siRNA into tumor cell capability and facilitated the escape of loaded siRNA from the endosome into the cytoplasm, leading to remarkable and specific gene knockdown efficacy in cancer cells, without toxicity.

5.2.2. Cells

Another strategy for TERM is the combination of scaffolds with cells, differentiated or undifferentiated, that can be implanted into the body to repair/regenerate a defect. For that, it is crucial to design a scaffold that will promote nutrients/metabolites diffusion and equal distribution of cells along to the center and inner surfaces of the scaffolds, since most of the cells have low capacity of invasion. Cellular attachment and proliferation is dependent on the scaffold architecture, such as material crystallinity, porosity, pores size, and pore interconnectedness. Figure 22 shows that cells seeding on microporous or microfibrous scaffolds flatten and spread as in two-dimensional systems, while nanoscale fibrous scaffolds increase cell binding.
sites due to their higher surface area to volume ratio for the adsorption of proteins and binding of ligands. Cells cultured on nanostructured surfaces can form a more orderly layer than on flat surfaces, and can make higher zone contacts improving cell attachment and cellular interactions. Scaffold porosity as well as the pores size can control the process of cells adhesion and migration to the surfaces of the scaffolds, and interconnecting pores allow cell growth inside the scaffold and nutrient flow.\(^{243}\) Reviews for cell responses to surface and architecture of tissue engineering scaffolds have been reported elsewhere.\(^{244}\)

The scaffold design is clearly an important contributing factor for the cell attachment and proliferation, but it is also necessary for the cells to be viable, produce an ECM and maintain normal tissue homeostasis. Different cell sources have been used to evaluate the cytotoxicity, compatibility, and cellular activities of the nanocomposites, such as osteoblasts, fibroblasts, hMSCs, human bone marrow stromal cells (HBMSCs), bone marrow-derived stem cells (BMSCs), and human endometrial stem cells (HESCs).\(^{21,35,103,245–247}\) Fricain et al.\(^{245}\) evaluated in vitro pullulan and dextran/nano-HAp composite scaffolds with HBMSCs seeding for bone regeneration. Results showed that the scaffolds induced the formation of multicellular aggregates and expression of early and late bone specific markers with HBMSCs seeding for bone regeneration. Results showed that the scaffolds induced the formation of multicellular aggregates and expression of early and late bone specific markers with HBMSCs in medium deprived of osteoinductive factors. Yan et al.\(^{103}\) used mouse lung fibroblasts (L929 cell line) seeded in SF/nano-CaP scaffolds to investigate the non-cytotoxicity of the scaffolds. Venugopal et al.\(^{246}\) prepared a chitosan/HAp nanofibrous composite scaffold with enhanced human fetal osteoblasts (hFOBs) proliferation for bone tissue engineering. Liu et al.\(^{248}\) demonstrated that a nanocomposite scaffold of HAp/chitosan can promote bone regeneration by supporting the adhesion, proliferation, and activating of integrin-BMP/Smad signaling pathway of BMSCs. Another report showed that nanocomposite scaffolds based on gelatin and CaP can support the attachment and proliferation of differentiated HESCs-derived osteoblast-like cells as can be seen in Figure 23.\(^{247}\) The presence of osteopontin, osteonectin and ALP mRNA after differentiation in the HESCs was also confirmed.

### 6. In Vivo Studies of Natural Polymer/CaP Nanocomposites in Tissue Engineering and Regenerative Medicine

Up to now, several animal models, such as rats, mice, rabbits, sheep, dogs and goats, have been used for TERM studies. Table 4 summarizes the recently reported in vivo studies performed on nanocomposites from biopolymers and CaPs.

Among different animal models used to evaluate the in vivo behavior of nanocomposite scaffolds, rat is the most used one, mainly because it is a relatively inexpensive model to establish. Also, they can reproduce fast, which enable the study of the function of particular genes during a reasonable period of time.\(^{251}\) Figure 24 shows a typical image section stained with the Masson’s trichrome of a SF/nano-CaP scaffold after implantation in rat femur defect for 3 weeks.\(^{38}\) It can be seen bone growth in the porous structure of the scaffold, with about 45% of new bone area, as well as no chronic inflammation.

Concerning human clinical studies, FDA or European Medicines Agency (EMEA) institutions must approve the release of biomaterial products before being employed in clinical applications. Consequently, only one pilot clinical trial study of natural polymers/CaP nanocomposites for the treatment of osteochondral lesions has been reported so far.\(^{252}\) A gradient tri-layer nanocomposite osteochondral scaffold based on collagen Type I/HAp, obtained by nucleating collagen fibrils with HAp nanoparticles has been implanted in the subchondral bone of 13
patients (15 defect sites) during 6 months. The scaffold cartilaginous layer consists of collagen, the intermediate layer consists of 60% collagen and 40% HAp, and the bone layer is composed of 30% collagen and 70% HAp. Histological analysis, although performed in only two cases, revealed the presence of perfectly formed subchondral bone and entirely scaffolds reabsorption. Nevertheless, it would be necessary to do follow-up studies of such procedure, especially regarding the histological quality of cartilage repair in the long term, in order to draw proper conclusions on its clinical effectiveness. Additional systematic analyses are therefore needed so that the clinical and morphological outcomes are well evaluated when compared with other alternative treatments such as bone-marrow stimulation techniques, mosaicplasty and autologous chondrocyte transplantation.

7. Concluding Remarks

Nanocomposite scaffolds combining biopolymers and nanosized CaPs have a great potential in TERM, due to their ability to mimic the structural and mechanical properties of native tissues. Natural polymers are appealing owing to their different degradation rates, whereas CaPs offer the required osteoconductivity and biocompatibility features.

The great research efforts for designing an ideal nanocomposite material for the repair and regeneration of damaged/diseased tissues have revealed the promise of nanocomposites comprising collagen, gelatin, silk fibroin, chitosan, alginate, hyaluronic acid, gellan gum, and derivatives as natural polymeric matrices, and HAp and β-TCP as bioactive fillers. Biomimetic strategies to produce nanocomposites for tissue engineering scaffolding, with microporosity and tunable surface, focused on 3D porous scaffolds, hydrogels, and nanofibrous scaffolds. The latter has shown several advantages due to its fibrous nano- to micro-architecture, which can mimic the network of collagen fibrils, homogeneous fiber size, high porosities, and pore distribution.

CaP nanopowders/nanoparticles have been prepared through wet chemical precipitation (or aqueous precipitation), sol-gel synthesis, hydrothermal synthesis, mechanochemical synthesis, microwave processing, and spray-drying methods. The most used technique is wet chemical owing the homogeneity of

Table 4. Recent in vivo studies of natural polymer/CaP nanocomposite scaffolds.

<table>
<thead>
<tr>
<th>Nanocomposite scaffolds</th>
<th>Animal model</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan/nanofibrous Hap</td>
<td>SD rat cranium defect</td>
<td>Bone regeneration in vivo after 10 and 20 weeks of implantation</td>
<td>[248]</td>
</tr>
<tr>
<td>SF/nano CaP</td>
<td>Wistar rat femur defect</td>
<td>New bone ingrowth after 3 weeks of implantation, without inflammatory response</td>
<td>[38]</td>
</tr>
<tr>
<td>PEG–PCL–PEG copolymer/collagen/ nano HAp</td>
<td>Rat muscle</td>
<td>Slight inflammatory response at 7 days post-surgery due to the degradation of the implant, indicating a good biocompatibility and biodegradability.</td>
<td>[162]</td>
</tr>
<tr>
<td>Pullulan and dextran/ nano-HAp</td>
<td>Rat femoral condyle</td>
<td>Dense mineralized tissue increasing from 15 to 90 days of implantation</td>
<td>[245]</td>
</tr>
<tr>
<td>PEG–PCL–PEG copolymer/collagen/ nano HAp</td>
<td>New Zealand white rabbit cranial defect</td>
<td>New bone tissue formation initially from the edge of the defects and the surface of the native bone and grew towards the center</td>
<td>[162]</td>
</tr>
<tr>
<td>HAp/SF</td>
<td>Rabbit</td>
<td>New bone formation</td>
<td>[249]</td>
</tr>
<tr>
<td>Calcium sulfate hemihydrate/ collagen/ nano-HAp</td>
<td>Rabbit femoral condyle</td>
<td>New bone formation after 12 weeks of implantation.</td>
<td>[250]</td>
</tr>
<tr>
<td>Pullulan and dextran/ nano-HAp</td>
<td>Goat mandibular defect</td>
<td>Dense mineralized tissue after 6 months of implantation. Dense lamellar collagen network</td>
<td>[245]</td>
</tr>
<tr>
<td>Pullulan and dextran/ nano-HAp</td>
<td>Goat tibial ostectomy</td>
<td>Dense mineralized tissue and regeneration of cortical bone after 6 months of implantation.</td>
<td>[245]</td>
</tr>
</tbody>
</table>

Figure 24. Microscopy images of histological Masson’s trichrome stained section of SF/nano-CaP scaffold after implantation in rat femur defect for 3 weeks. S: scaffold residuals; B: formed new bone; M: bone marrow; R: rapid forming new bone. Scale bars: a) 200 µm and b) 100 µm. Adapted with permission. [38] Copyright 2013, SAGE.
the final product and the easiness of parameters control during the synthesis. Conventional technologies used to fabricate the nanocomposite scaffolds include foam replica, solvent casting and particulate-leaching, freeze-drying, gas foaming and phase separation. Rapid prototyping and electrospinning are more sophisticated techniques for the production of 3D structures and fibers, respectively, with the ability to mimic new tissue structures and the possibility of incorporating pharmaceutical agents. Additionally, molecular self-assembly is used to produce nanofibrous by creating supramolecular architectures.

Finally, nanocomposites from natural polymers and CaPs hold nano-featured structures, such as high surface area and enhanced porosity that are a must for the appropriate cellular adhesion, proliferation, and differentiation. Furthermore, they can be functionalized with bioactive molecules and stem cells in order to enhance tissue healing/regeneration. In vitro cell culturing in 3D using perfusion bioreactor systems may be also applied for producing mature tissues. Such dynamic systems can provide an optimal environment for convectively transporting nutrients to cells and remove metabolites, with appropriate mechanical stresses, to guide cell growth and proliferation, and extracellular matrix production.

Succinctly, it can be concluded that the perfect natural nanocomposite scaffold, mimicking the hierarchical structure and morphology of bone while presenting a temporary function, has not yet been developed. Still, silk fibroin and collagen biopolymers combined with CaPs showed great promise in pre-clinical studies. These scaffolds are still in stages of research and development, lacking application in clinical surveys. Future developments of this kind of nanocomposites for tissue repair and regeneration, towards clinical applications, should be devoted on the clear understanding of the nanocomposite-tissue interactions, on the optimization of their composition and hierarchical structure for long-term service, and the related mechanical strength, especially the fatigue limit under periodic external stress. Besides, the use of these nanocomposites with therapeutic effect and drug delivery, combined with differentiated or undifferentiated autologous cells, should be deeply investigated.

Acknowledgements
The research leading to this work has received funding from the European Union’s Seventh Framework Programme (FP7/2007–2013) under grant agreement n° REGPOT-CT2012–316331-POLARIS, and from QREN (ON.2 – NORTE-01–0124-FEDER-000016) cofinanced by North Portugal Regional Operational Program (ON.2 – O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF).

Note: Figure 6, 7 and 12 were replaced after initial publication online.

Received: July 25, 2014
Revised: October 14, 2014
Published online:


[40] C. Tanase, A. Sartoris, M. Popa, L. Verestruic, R. Unger, C. Kirkpatrick, Biomaterials 2013, 8, 025002.


[63] P. He, K. Ng, S. Toh, J. Koh, Biomacromolecules 2012, 13, 2692.


