Chapter X

Biomimetic Strategies to Engineer Mineralised Human Tissues

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Abstract

In the last few years, many reports have been describing promising biocompatible and biodegradable materials that can mimic in a certain extent the multidimensional hierarchical structure of bone, while are also capable of releasing bioactive agents or drugs in a controlled manner. Despite these great advances, new developments in the design and fabrication technologies are required to address the need to engineer suitable biomimetic materials in order tune cells functions, i.e. enhance cell-biomaterial interactions, and promote cell adhesion, proliferation, and differentiation ability. Scaffolds, hydrogels, fibres and composite materials are the most commonly used as biomimetics for bone tissue engineering. Dynamic systems such as bioreactors have also been attracting great deal of attention as it allows developing a wide range of novel *in vitro* strategies for the homogeneous coating of scaffolds and prosthesis with ceramics, and production of biomimetic constructs, prior its implantation in the body. Herein, it is overviewed the biomimetic strategies for bone tissue engineering, recent developments and future trends. Conventional and more recent processing methodologies are also described.

X.1 Introduction

Nowadays, the development of new biomaterials for bone tissue engineering has made important contribution to modern health care. Each biomaterial has specific chemical, physical, mechanical and biological properties, important for the behaviour and outcome of the implant.

Autografts and allografts have been used to repair bone fractures and other defects, however concerns include risk of disease transfer, infection, potential immunogenicity, and insufficient supply (Finkemeier 2002).

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Biomimetic materials have been attracting considerable attention owing to their ability to mimic the structural, mechanical, and biological properties of natural tissues. The major difficulty in tissue engineering arises from the specificity of the tissue that it is proposed to treat, compliance of patients and other related restrictions. A deeply progress has been made in designing and processing of new materials in order to properly address cell activity. This is an important issue to be considered as regeneration processes involves achieving the desired cell functioning, i.e. stimulate specific cellular responses and activate genes that stimulate cells differentiation and extracellular matrix production for enhancing the regeneration of the damaged tissues. In case of bone, it is required three-dimensional constructs hierarchically structured that can comprise several levels of organization, i.e. from the macroscopic tissue arrangement down to the molecular arrangement of proteins (Aizenberg et al. 2005). These hierarchical structures also provide improved mechanical performance and allow a suitable transduction of the mechanical stimuli to the cellular level (Sprio et al. 2011).

Different materials have been exploited for scaffolds fabrication, attending diverse need in bone tissue engineering, such as natural-based and synthetic polymers, inorganic/ceramic materials, and composites. Polymers have great design flexibility and mechanical strength. Polymers from natural origin offer the advantage of being similar to the extracellular matrix, and do not cause chronic inflammation or immunological reactions and toxicity (Mano et al. 2007). Ceramics such as hydroxyapatite ($Ca_5(PO_4)_3OH$, HA) and β-tricalcium phosphate ($Ca_3(PO_4)_2$, β-TCP) are the most used in the clinics due to their good osteoconductivity, biocompatibility, and bioresorbability (Bohner 2000). However, ceramic materials have low elasticity and high brittleness, resulting in poor mechanical strength, which can be solved by combining them with polymers as composite materials (Oliveira et al. 2009c; Yan et al. 2013).

In the next sections, it are discussed some of the current reported biomimetic strategies for bone tissue engineering. A special focus is given on new developments for designing and processing of biomimetic materials.

X.2 Biomimetic Strategies for Bone Tissue Engineering

Among the biomimetic materials that have been developed to engineer bone tissue scaffolds, hydrogels, fibres and composites 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. Considerations of scaffold design include the shape of the original tissue, mechanical properties, ability to direct cell–matrix and cell–cell interactions, and porous structures for efficient mass transport. Materials used to engineer these tissues are of great importance because it also must be biocompatible and degrade at a rate matching that of the new tissue formation.

X.2.1 Scaffolds

Scaffolds are defined as three-dimensional (3D) porous solid biomaterials designed to fulfill basic requirements, namely: (i) to promote cell-biomaterial interactions, cell adhesion, growth and migration, and ECM deposition, (ii) to permit sufficient transport of gases, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation, (iii) degrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest, (iv) to elicit a minimal degree of inflammation or toxicity *in vivo*, and (v) possess adequate mechanical properties necessary to temporarily offer structural support until the formation of new tissue occurs (Langer and Tirrell 2004).

Scaffolds targeted for bone tissue repair and regeneration can be produced from natural and synthetic polymers and bioactive ceramics, and their combinations, with specific pore size, porosity, surface-area-to-volume ratio and crystallinity. Technologies applied for scaffolds fabrication include sponge replica method (Oliveira et al. 2009b), solvent casting and particulate-leaching (Hou et al. 2003), freeze-drying (Liapis et al. 1996), phase separation (vandeWitte et al. 1996), gas foaming (Dehghani and Annabi 2011), and rapid prototyping (RP) (Abdelaal and Darwish 2011).

Several studies have been reported concerning scaffolds design, characterization and biological evaluation (Abdelaal and Darwish 2011; Azami et al. 2012; De Nardo et al. 2012; Fabbri et al. 2010; Oliveira et al. 2009b; Oliveira et al. 2009c; Oliveira et al. 2006b; Yan et al. 2013). For example, Columbus et al. (Columbus et al. 2013) have investigated the feasibility of varying the pore size of poly ε-caprolactone (PCL) scaffolds by altering the molecular weight of the porogen and studied the effect of degradation on morphological characteristics and mechanical properties of scaffolds by correlating to the extent of degradation. The authors observed that degradation resulted in scaffolds with narrower pore size distribution have better retention of morphological and mechanical characteristics as compared to scaffolds with broader distribution.

Our group has been developing porous structures for bone tissue engineering using diverse materials and techniques. Oliveira et al. (Oliveira et al. 2009b) developed hydroxyapatite (HA) single scaffolds with a porosity of ~70%, and macropores diameter in the range of 50–600 μm with high pore uniformity and interconnectivity across the scaffolds by means of sponge replica method (Fig. X.1).

Fig. X.1 Macroscopic appearance of the HA scaffolds after sintering at 1300 °C
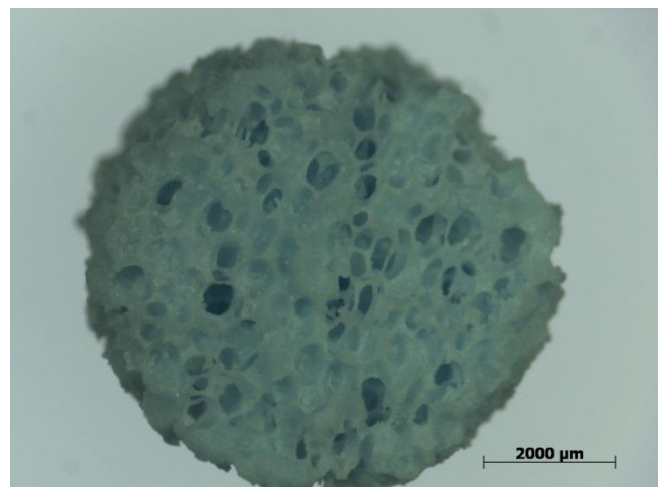
Oliveira et al. (Oliveira et al. 2012) developed semi-rigid 3D tubular porous structures consisting of starch/PCL, filled by a gellan gum hydrogel concentric core aimed at inducing the regeneration within spinal-cord injury sites obtained through RP technology. It was demonstrated that bone-like apatite layers could be produced on the surface and interior of the constructs and the mineralization process was controlled by the surface, which acted as a template for the nucleation and growing of the apatite layer. Lima et al. (Lima et al. 2013) developed a bottom-up approach that allows the construction of 3D biodegradable scaffolds from PCL and starch microfabricated membranes, by means of micro hot-embossing. In addition, it was shown that the human bone marrow stem cells (hBMSCs) proliferated and maintained the expression of the stromal progenitor marker STRO-1 when cultured on both PCL and SPCL microfabricated membranes.

An important consideration for biomimetic materials engineering is their biocompatibility capacity. *In vitro* bioactivity and biocompatibility assays include mineralization tests in simulated body fluid (SBF) and the use of adequate cell cultures to induce cell adhesion, growth, proliferation and differentiation (Rumian et al. 2013; Yang et al. 2013; Wang et al. 2013; Azami et al. 2012), respectively. For example, adipose derived stem cells (ASCs) with multi-lineage differentiation capacities have been demonstrated as an alternative cell candidate for bone regeneration (Zreiqat 2013). It was reported that biomimetic constructions of undifferentiated rabbit ASCs with fully interconnected porous β-TCP scaffolds encapsulated by collagen hydrogel enhanced osteogenesis in a critical-sized defect of rabbit radii (Yang et al. 2013).

Scaffolds have also been developed with particular interest for bone and osteochondral tissue engineering applications, where both underlying bone and cartilage tissues are damaged. Therefore, a strategy for these applications is the construction of bilayered scaffolds consisting of both osteogenic and chondrogenic regions, which can be manufactured in a single integrated implant or fabricated independently and joined together with sutures or sealants. Thus, bilayered scaffolds are able to promotes simultaneous regeneration of two tissues, bone and cartilage with different properties and biological requirements (Oliveira et al. 2006b; Yan et al. 2013). For example, Oliveira et al. (Oliveira et al. 2006b) fabricated a HA/chitosan bilayered scaffold by combining a sintering and a freeze-drying technique, aiming the regeneration ofosteochondral defects. The bone region of the scaffold was composed of porous sintered HA and produced through sponge replica method. The cartilage region was composed of chitosan poured onto the top of the HA scaffold, previously prepared, followed by freeze-drying technique. In that work, it was demonstrated through *in vitro* cell studies that both HA and chitosan layers provided an adequate support for cell attachment, proliferation and differentiation into osteoblasts and chondrocytes, respectively.
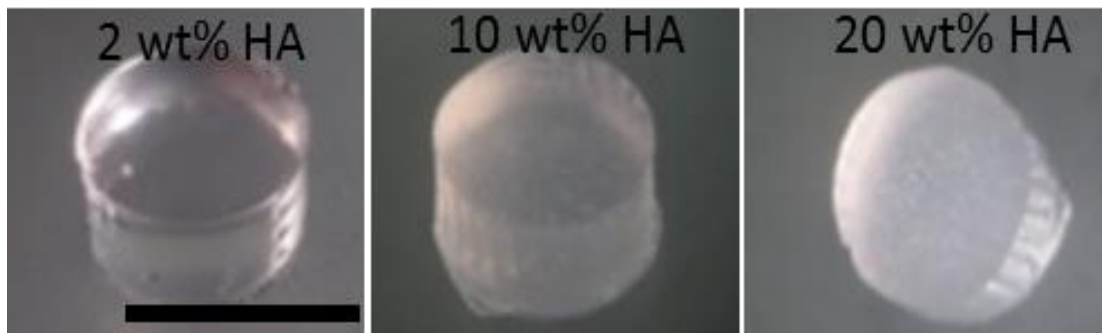
X.2.2 Hydrogels

Hydrogels are water-swollen cross-linked hydrophilic polymer 3D networks to form insoluble polymer matrices, which is soft and elastic due to their thermodynamic compatibility with water (Slaughter et al. 2009; Zhu and Marchant 2011). Hydrogels can be classified into physical and chemical hydrogels according to their cross-linking mechanism (Chung and Park 2009; Slaughter et al. 2009). Physical cross-links keep the hydrogel from dissolving in water due to the entangled chains, hydrogen bonding, hydrophobic interaction and crystallite formation. Chemical cross-links are permanent junctions formed by covalent bonds, which can be created by polymerize end-functionalized macromers (Liu et al. 2010; Nguyen and West 2002). Hydrogels can also be classified by their ionic charge (neutral, cationic, anionic and ampholytic), structure (amorphous, semicrystalline and hydrogen-bond) and preparation methods (homopolymer, copolymer, multipolymer and interpenetrating polymer network) (Slaughter et al. 2009). 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 (Slaughter et al. 2009). 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 (Hoffman 2002). An innovative alternative to the classical techniques to characterize cross-linked hydrogels has been reported. High-resolution magic angle spinning (HR-MAS) NMR spectroscopy enables to both characterize and quantify any unreacted cross-linkable moieties present in a chemically cross-linked, swollen hydrogel network (Van Vlierberghe et al. 2010). Most hydrogels are injectable allowing homogeneous seeding of cells throughout the scaffolds and formation of hydrogels *in situ* (Tan and Marra 2010; Kretlow et al. 2007).

There are three major classes of hydrogels, based on the polymer origin: natural, synthetic, or natural/synthetic hybrids. Natural hydrogels are derived from nature and have been used due to their biocompatibility, inherent biodegradability and critical biological functions. Nevertheless, the use of natural hydrogels is often restricted because of purification, immunogenicity, pathogen transmission and poor mechanical properties. On the other hand, synthetic hydrogels possess more reproducible chemical and physical properties and it can be molecularly tailored with mechanical strength and biodegradability. Moreover, hydrogels porosity may be controlled by solvent casting/particulate leaching, phase separation, gas foaming, solvent evaporation, freeze-drying, and blending with non-crosslinkable linear polymers. Excellent reviews regarding a depth description of natural and synthetic hydrogels are referred (Oliveira and Reis 2011; Van Vlierberghe et al. 2011).

Synthetic polymer derived hydrogels are not always able to provide an alternative to natural-based polymers. Thus, hydrogels can be assembled through copolymerization or combined with additional synthetic or natural polymers, to create semisynthetic materials with desired physical properties, reproducibility, and biological activity. To date, natural polymers, such as collagen, fibrinogen, hyaluronic acid, chitosan and heparin, have been used to make hybrid hydrogels with synthetic polymers, such as poly ethylene glycol (PEG), poly *N*-isopropylacrylamide and poly

vinyl alcohol (PVA) (Hiemstra et al. 2007; Wang et al. 1999; Bryant et al. 2004). The hybridization can occur at a molecular level depending on the size and nature of building blocks. Our group is a pioneer in processing hydrogels based on a polysaccharide gellan gum, obtained from *Sphingomonas elodea*, for tissue engineering applications (Oliveira et al. 2010; Pereira et al. 2011; Silva-Correia et al. 2011; Silva-Correia et al. 2013). Methacrylated gellan gum hydrogels were produced through chemical modification by means of methacrylation of low acyl gellan gum. Gellan gum-based hydrogels (Fig. X.2) can be tunable to enhance the mechanical properties, allow to control endothelial cells infiltration and blood vessel ingrowth's, and can be formed in situ in few minutes (Silva-Correia et al. 2011). Recently, gellan gum-based hydrogels have also been proposed for bone and osteochondral applications by means of blending with hydroxyapatite particles (Manda-Guiba et al. 2012; Pereira et al. 2014). The composite showed superior mechanical properties as compared to the polymeric hydrogel, while the optimization of the HA content in the composite possibly tuning its bioactivity.

Fig. X.2 Macroscopic appearance of the methacrylated ellan gum hydrogels with different HA content, after ionic crosslinking (scale bar = 5 mm)

Hydrogels have been used as scaffolds to provide bulk and mechanical constitution to an engineered tissue, for cellular organization and morphogenic guidance. As aforementioned, it is desirable to synthesize scaffolds to mimic the structure and functions of ECM (Tibbitt and Anseth 2009; Zhu and Marchant 2011). Numerous bioactive peptide sequences derived from ECM proteins such as fibronectin, laminin and collagen, have been incorporated into synthetic hydrogels. In addition, cellular attachment, proliferation and growth in hydrogels can be promoted with the inclusion of arginine–glycine–aspartic acid (RGD) peptide sequence (Shin et al. 2003). This cellular adhesion and proliferation is assured by controlling the rate of biodegradation of the hydrogel scaffolds, which is achieved by clearing the implanted hydrogel based on a predetermined rate of hydrolytic breakdown of ester bonds in the polymer network. Cells encapsulated in hydrogels during the cross-linking process have been produced and characterized for drug delivery and tissue engineering applications (Rada et al. 2013).

X.2.3 Fibres

Fibrous scaffolds are a good option to mimic the fibrous structure present in native ECM owing their high porosities, isotropic structures, and homogeneous fibre size and pore distribution. Porosity is interesting for various applications, namely for the entrapment of bioactive molecules. The mechanical properties of fibrous scaffolds are dependent on their composition, fibre diameter and orientation (Vatankhah et al. 2013). Fibre diameter can be reduced with synthetic polymers blended with proteins (Thomas et al. 2009). Furthermore, nanoscale fibrous scaffolds with well-controlled patterned structures have received particular interest to enhance cell functions as cell adhesion, migration, proliferation and differentiation. Nonwoven fibrous matrices with micro-sized fibres possess isotropic structure with a high porosity and mechanical strength, and good chemical stability (Ng et al. 2012).

Fibrous scaffolds design comprises natural and synthetic polymers. Natural polymers recently used for bone tissue engineering include collagen, gelatin, silk fibroin, elastin, fibrinogen, hyaluronan, and chitosan, due their high biological recognition (Mano et al. 2007). For example, PCL is successfully processed with gelatin for obtaining a nanofibre network with random fibre distribution, good biocompatibility with mesenchymal stem cells in terms of adhesion and proliferation (Alvarez Perez et al. 2012). Blends of starch-polycaprolactone (SPCL) have also been used to produce scaffolds by fibres meshing (Gomes et al. 2008). Interestingly, Oliveira et al. (Oliveira et al. 2011; Oliveira et al. 2008) shown that SPCL scaffolds (Fig. X.3) supported BMSCs osteogenic differentiation *in vitro* and de novo bone formation *in vivo*.

Fig. X.3 Microscopic appearance of the SPCL scaffolds

1000 µm

Fabrication techniques as molecular self-assembly, phase separation, and electrospinning have been used for fibrous scaffolds production. Molecular self-assembly is able to produce highly ordered nanofibrous but it is limited to molecules for self-assembly. Phase separation method can

only produce randomly distributed fibres in the sub-micron range. Electrospinning can generate fibres, from nano- to micro-size, with controllable pore size, fibre size and stiffness, and matrix turnover (Hong and Madihally 2011). Electrospun fibre scaffolds for bone tissue engineering have been recently reported (Cheng et al. 2013; Kolluru et al. 2013; Alvarez Perez et al. 2012). Diverse cell types have been applied for electrospun fibres *in vitro* studies, as neural stem cells (Binan et al. 2013), chondrocytes (Li et al. 2013), cardiomyocytes (Balasubramanian et al. 2013), osteoblast-like cells (Bock et al. 2013) and mesenchymal stem cells (Ni et al. 2011; Jia et al. 2013).

The incorporation of bioactive agents to electrospun fibres can lead to enhanced biomimetic scaffolds. It has been demonstrated that cell-substrate interaction is strongly affected by the presence of chemical cues, able to support cell adhesion, proliferation and differentiation (Stankus et al. 2008).

The combination of electrospun fibres and hydrogels scaffolds can create synergistically superior structures to therapeutic use within the tissue engineering community (Bosworth et al. 2013).

X.2.4 Composites

Composite materials appeared as a strategy to mimic the human bone, which is a 3D composite material, composed of organic, inorganic and cellular phases, strictly assembled to form the natural tissue. Composites are considered as a combination of two or more materials, with different compositions and properties, resulting in a single structure with significantly improved mechanical and biological properties. Therefore, composites involving a polymeric matrix and bioactive inorganic fillers (Fig. X.2) have been developed as promising approach for bone tissue engineering applications.

Composites are most frequently manufactured from: (i) natural origin, like polysaccharides (cellulose, chitin, glycosaminoglycans) and proteins (collagen, silk, fibrinogen, elastin) owing their similarities with the extracellular matrix, chemical versatility, and good biological performance (Mano et al. 2007); (ii) synthetic biodegradable polymers as polylactic acid (PLA), polyglycolic acid (PGA), its copolymer polylactic-co-glycolic acid (PLGA), and PCL (Blasier et al. 1997; Bucholtz et al. 1994; Causa et al. 2006; Wei et al. 2009); and (iii) bioactive ceramic materials, such as HA, β-TCP, calcium silicate and phosphate-based glasses, due to its biocompatibility, osteoconductivity and degradation rate (Daculsi et al. 2003; Bohner 2000). There is a range of possible combinations capable of producing composites with enhanced properties (Bhumiratana et al. 2011; Hunter and Ma 2013; Lu et al. 2011; Popescu et al. 2013; Oliveira et al. 2009c). For example, the incorporation of HA in silk sponge matrices was shown to enhance the osteoconductivity and mechanical properties of the scaffolds (Bhumiratana et al. 2011). Wang et al. (Wang et al. 2011) created biomimetic matrices using genetically engineered elastin-like polypeptides to construct mechanically robust elastin/HA composites.

Regarding the production of composite scaffolds, first of all it is essential to achieve good compatibility between the phases while keeping the porous structure and the mechanical properties of the scaffolds. Interestingly, it has been attempted the production of hierarchical nanocomposite scaffolds (Fig. X.4). Our group synthesized calcium phosphates in silk fibroin by using an *in situ* synthesis method, where phosphate ions are added into the calcium chloride solution with dissolved silk fibroin, followed by salt-leaching/freeze-drying techniques to obtain the scaffolds (Yan et al. 2013; Oliveira et al. 2006a). Kim et al. (Kim et al. 2008) prepared aqueous-derived silk fibroin scaffolds with the addition of polyaspartic acid, followed by the controlled deposition of calcium phosphate by exposure to chloride and sodium phosphate monobasic solutions. In another study, HA micro-particles were embedded in silk sponges to generate highly osteogenic composite scaffolds capable of inducing the formation of tissue-engineered bone (Bhumiratana et al. 2011).

In addition, the use of powder fillers from nano- to macro-size ranges with hierarchical structures have attracted special attention since it can easily mimic the structural and mechanical properties of natural tissues (Engel et al. 2008). The nanopowders often act as physical cross-links to the polymer chains, reinforcing the mechanical properties of the nanocomposites (Smith et al. 2009). Yan et al. (Yan et al. 2013) prepared a composite scaffold made from silk fibroin and nano-sized calcium phosphates and it was obtained calcium phosphate particles with size less than 200 nm and a uniform distribution in the scaffolds. Zhou et al. (Zhou et al. 2013) fabricated electrospun fibrous bio-nanocomposite scaffolds reinforced with cellulose nanocrystals by using maleic anhydride grafted PLA as matrix, with improved thermal stability and mechanical properties.

A biocomposite of nano-sized calcium silicate and PCL was fabricated using nano-calcium sulphate slurry in a solvent-casting method (Wei et al. 2009). It was showed that the hydrophilicity, compressive strength, and elastic modulus of n-CS/PCL composites were improved, as well as the *in vitro* bioactivity. The apatite layers that formed on the composite surfaces facilitated cell attachment and proliferation. Similar results were obtained by Kotela et al. (Kotela et al. 2009).

Electrospun nanocomposite scaffolds containing a polymer matrix and nano-sized HA supported cell attachment, proliferation and differentiation, and are thus considered very promising tissue engineering (Venugopal et al. 2008; Pascu et al. 2013; Chang et al. 2013; Li et al. 2012).
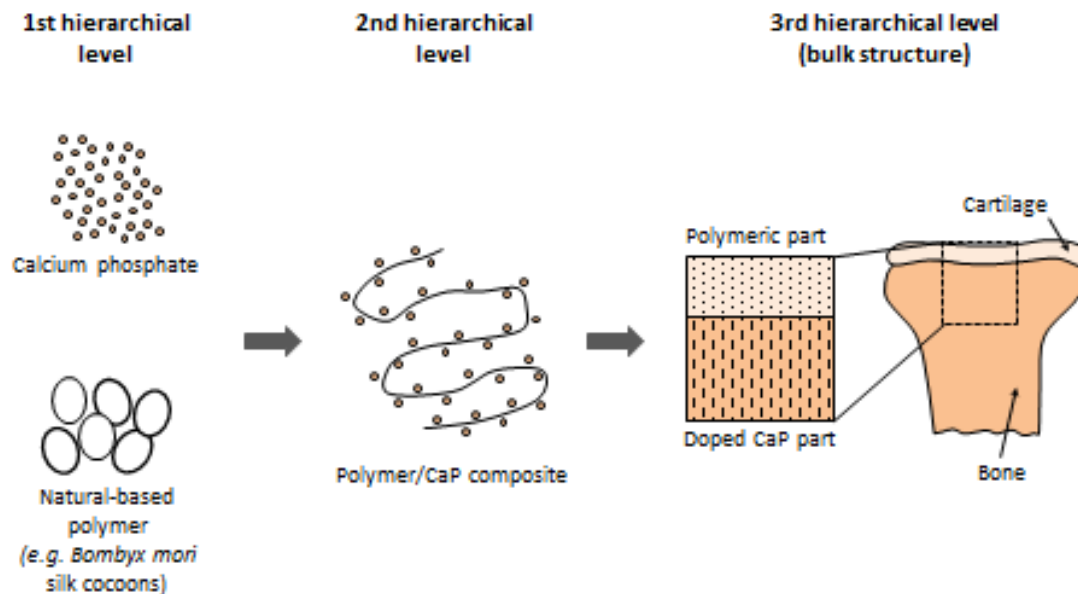
Fig. X.4 Schematic diagram of a hierarchical nanocomposite scaffold fabricated from polymer and calcium phosphates

X.2.5 Dynamic systems

For over two decades, *in vitro* mineralization assays have been used as a biomimetic route to evaluate the bioactivity and biocompatibility of biomaterials. The common *in vitro* studies are performed by immersing the materials in SBF at static conditions as proposed by Kokubo et al. (Kokubo and Takadama 2006). However, the *in vitro* culture techniques have nutrient and mass transfer limitations that must be overcome to increase the feasibility of cell-based tissue engineering strategies. Thus, an *in vitro* biomimetic approach including dynamic studies using bioreactor systems have been used to relieve these limitations by continuously mixing media and by convectively transporting nutrients to cells (Yeatts and Fisher 2011), with appropriate mechanical stresses. 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.

A wide array of bioreactor systems for bone tissue engineering have been developed, as spinner flasks (Wang et al. 2009), rotating wall vessels (Zhang et al. 2009), and perfusion systems (Oliveira et al. 2012). Spinner flask and rotating wall systems create a homogenous media on the exterior of the scaffold, while on perfusion systems, the media is perfused through the pores of

the scaffold, which enables local supply of nutrients providing a better control of the cell microenvironment (Gaspar et al. 2012).

Some authors have studied the mineralization of apatite layers under dynamic conditions using bioreactor systems (Oliveira et al. 2007; Yeatts et al. 2012; Lv et al. 2009; Gardel et al. 2013). Oliveira et al. (Oliveira et al. 2009a) showed that bone-like apatite layers have been formed on the surface and inside of starch/polycaprolactone 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 MSCs proliferation and osteogenesis for bone tissue engineering (Liu et al. 2012; Yeatts et al. 2013). For example, hMSCs cultured on nanofibrous electrospun poly(lactic-co-glycolic acid)/PCL scaffolds to implant into rat femoral condyle defects, showed a more rapid bone regeneration in defects implanted with bioreactor cultured scaffolds in comparison to defects implanted with statically cultured scaffolds (Yeatts et al. 2013).

X.3 Current processing methodology in biomimetic engineering

A wide range of conventional techniques have commonly been used for bone scaffolding applications such as, sponge replica method, solvent casting and particulate-leaching, freeze drying, gas foaming and phase separation. Rapid prototyping and electrospinning as more sophisticated techniques are also used for 3D structures and fibres, respectively. The conventional methods are often simple to design, inexpensive and flexible to optimize or modulate physicochemical properties. Despite being expensive and suffering from certain drawbacks of choice of materials and capital costs rapid prototyping and electrospinning techniques are extremely attractive in their ability to mimic new tissue structures and possibility of incorporating pharmaceutical agents. A brief description of each technique is provided as follows.

X.3.1 Sponge replica method

Sponge replica method is based in the impregnation of an aqueous suspension in porous synthetic polymeric (usually polyurethane) sponges until the total filling of the pores. The impregnated sponge is then passed through rollers or centrifuge in order to remove the excess suspension and left to dry (Oliveira et al. 2009b) After, the sponges are heated at temperatures between 400-900ºC for the diffusion of the polymeric template and a porous structure is obtained. Finally, the scaffolds are densified by sintering at high temperatures. This method can reach total open porosity levels within the range 40% – 95% and are characterized by a reticulated structure of highly interconnected pores with sizes between 200 μm and 3 mm (Studart et al. 2006).

X.3.2 Solvent casting and particulate-leaching

Solvent casting and particulate-leaching is a technique widely used to successfully fabricate 3D porous scaffolds (Thadavirul et al. 2013; Suh et al. 2002; De Nardo et al. 2012; Yan et al. 2013). 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 evaporated, resulting in the solidification of the polymer. Then, the salt crystals are leached away using water to form the pores of the scaffold. The pore size can be controlled by the size of the salt crystals and the porosity by the salt/polymer ratio. This leaching process has been applied to the development of porous scaffolds for the growth of endothelial cells (Cao et al. 2005). However, certain critical variables such as pore shape and interconnectivity of the pores are not controlled with this method (Moore et al. 2004).

X.3.3 Freeze-drying

Freeze-drying is based on dissolution of a polymer into a solvent with addition of water, followed by quickly frozen at different temperatures and rates (Silva et al. 2012). A porous polymeric structure is obtained after freeze-drying to remove the dispersed water and the solvent (Liapis et al. 1996). The porosity level of the foams is controlled by varying the freezing time and the annealing stage. The main difficulty associated with this process is to ensure structural stability and adequate mechanical properties of the porous constructs after hydration. This limitation hinders its use when the application involves conditions with mechanical stress. Moreover, pore size is relatively small and porosity is often irregular (Hottot et al. 2004).
Our group has used this method to develop porous single layered (Fig. X.4) and bilayered scaffolds (Oliveira et al. 2009b; Yan et al. 2013; Malafaya and Reis 2003).

Fig. X.4 Microscopic appearance of the chitosan freeze-dried scaffolds
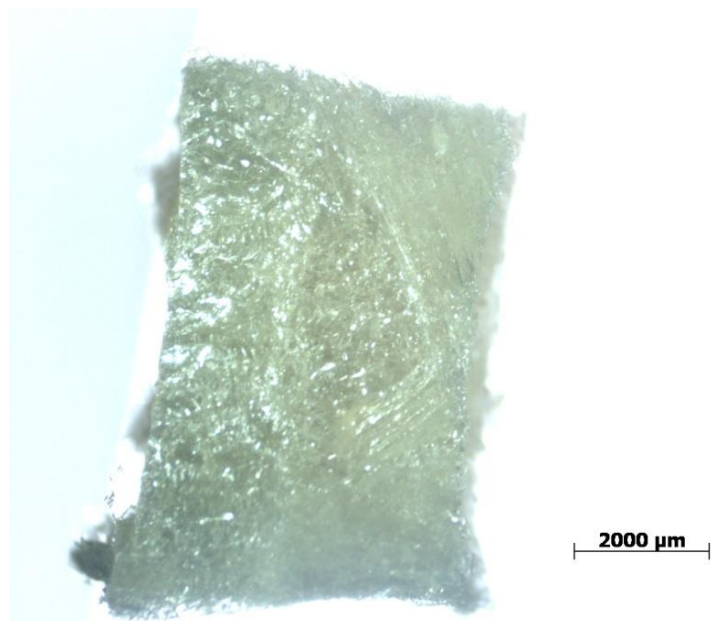
X.3.4 Gas foaming

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 foaming process, the porogen is removed and a highly interconnected pore structure is created (Nam et al. 2000). The porosity of the scaffolds depends of the amount of gas dissolved in the polymer. This technique does not require the use of organic solvents and high temperature as for the most fabrication techniques.

Porous polymeric foams prepared using gas foaming technique has emerged in recent years with application as scaffolds for bone tissue engineering (Floren et al. 2011; Beskardes et al. 2012; Salerno et al. 2010). Results indicate that the micro-architecture of the pore structure of the scaffolds plays a crucial role in defining cell seeding efficiency, proliferation, and osteogenic differentiation (Salerno et al. 2010).

X.3.5 Phase separation

Phase separation technique is based on the separation into more than one phase in order to lower the system 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 (Baker 2000) but has the disadvantage of forming pores with diameters on the order of a few to tens of microns and not uniformly distributed. However, controlled phase separation, as thermally induced phase separation, has been used for scaffold fabrication (Zhang and Ma 2001; Mandoli et al. 2010). This process is based on the decrease in solubility associated with temperature increase. After demixing is induced, the solvent is removed by extraction, evaporation or freeze drying. For example, Mandoli et al. (Mandoli et al. 2010) prepared highly porous scaffolds made of PLLA by thermally induced phase separation starting from 1,4-dioxane/PLLA solutions for applications in vascular nets and angiogenesis.

X.3.6 Rapid prototyping

Rapid prototyping (RP), also known as solid freeform fabrication or additive manufacturing, is an advanced technique used to design complex-shaped objects directly from computer data such as Computer Aided Design (CAD), Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) data (Abdelaal and Darwish 2011; Yeong et al. 2004). The fabrication process involves a 3D design of the scaffold, which is directly fabricated layer-by-layer. As a result, RP

technique is considered the best alternative for achieving precise control of pore size, geometry and interconnectivity, which enable cells to penetrate into the scaffold.

Robocasting is also an RP-based process in which a colloidal suspension is extruded through a micron-sized nozzle in a defined trajectory to form a 3D structure (Hoelzle et al. 2008). This technique has been used to fabricate porous β-TCP scaffolds with a controlled architecture, with enhanced compressive strength and toughness (Miranda et al. 2006).

X.3.7 Electrospinning

Electrospinning has received considerable interest for use in tissue engineering aimed at producing polymeric nanofibres non-woven membranes scaffolds to the order of nanometers with large surface areas and superior mechanical properties (Agarwal et al. 2008). 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 fibres. As this jet travels, the solvent evaporates and the nanofibres are deposited in the collector. The characteristics of the nanofibres depend on various properties of the solution and on the processing parameters Electrospinning provides a simpler and more cost-effective means to produce scaffolds with an inter-connected pore structure and fiber diameters in the sub-micron range when compared to others techniques like phase separation.

X.4 Final remarks and future trends

Biomimetic strategies for bone tissue engineering are growing exponentially over time towards the development of novel materials capable of mimicking the structural, mechanical and biological properties of native bone tissue. An ideal biomimetic material must also be biocompatible and degrade at a rate matching the ECM deposition rate. In addition, demanding for three-dimensional porous constructs hierarchically structured across a range of length scales arises from their unique structure-properties relationship. The use of dynamic systems, particularly perfusion bioreactors, to coat scaffolds with apatite layer is a key component for bone tissue engineering due to their ability of mass transfer and nutrients diffusion throughout the scaffolds.

Future developments of biomimetic materials for bone tissue engineering should be focused on the selection of materials, design and fabrication of composite scaffolds hierarchically nanostructured, with appropriate mechanical properties and that can stimulate cell-biomaterial interactions and promote cell adhesion, proliferation and differentiation.

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