

Chapter X

Biomimetic Strategies to Engineer Mineralised Human Tissues

Sandra Pina^{1,2}, Joaquim Miguel Oliveira^{1,2} and Rui L. Reis^{1,2}

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).

S. Pina(✉) • J.M. Oliveira • R.L. Reis

¹3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal;

²ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal.

e-mail: sandra.pina@dep.uminho.pt

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 ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$, HA) and β -tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$, 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

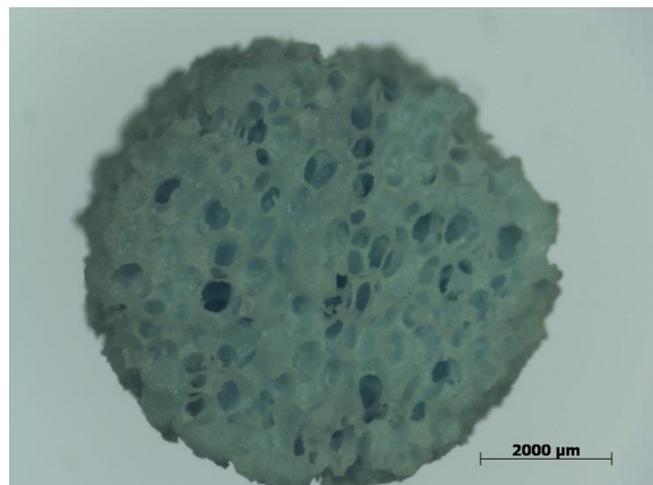
Scaffolds are defined as three-dimensional (3D) porous solid biomaterials designed to fulfill basic requirements, namely: (i) to promote cell-biomaterial interactions, cell adhesion, growth and migration, and ECM deposition, (ii) to permit sufficient transport of gases, nutrients, and regulatory factors 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 $\sim 70\%$, 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 promote 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 of osteochondral 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.

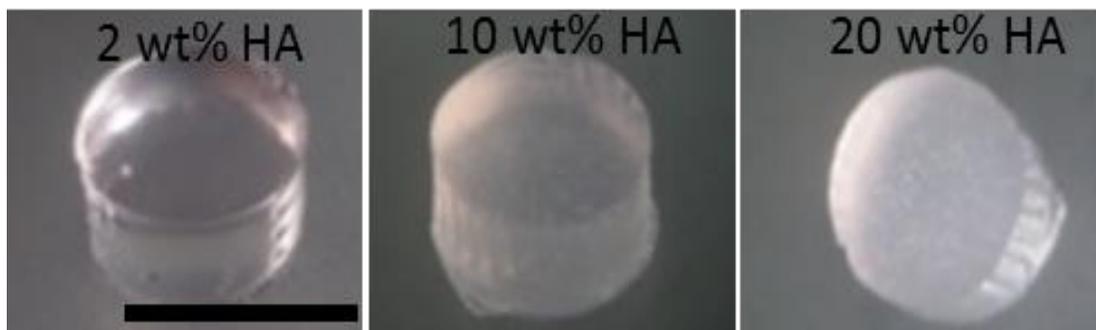


Fig. X.2 Macroscopic appearance of the methacrylated gellan gum hydrogels with different HA content, after ionic crosslinking (scale bar = 5 mm)

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



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 Madhally 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/HAComposites.

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).

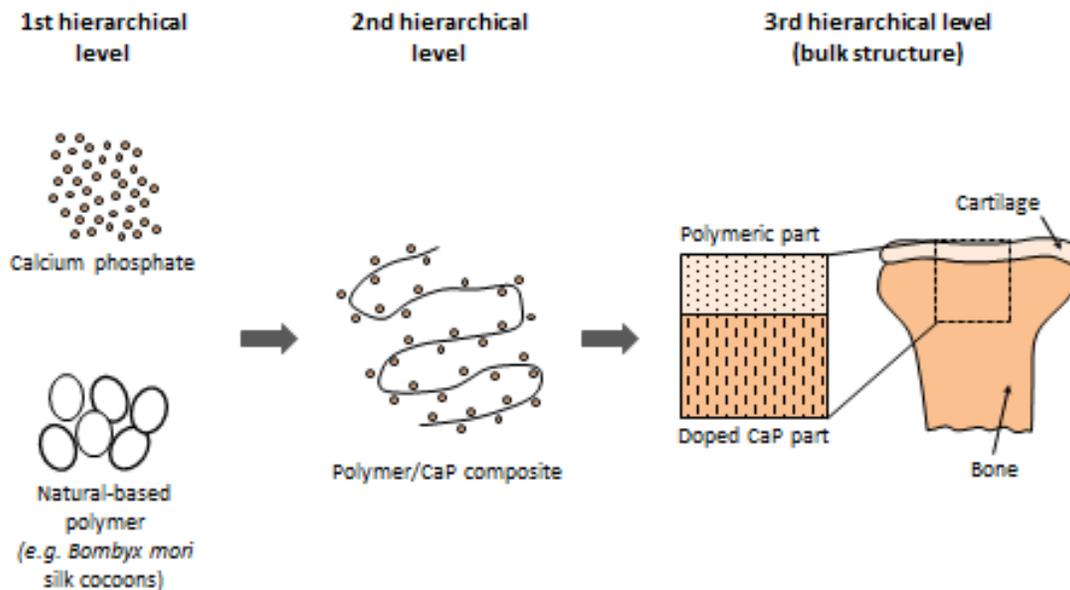


Fig. X.4 Schematic diagram of a hierarchical nanocomposite scaffold fabricated from polymer and calcium phosphates

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 homogeneous 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 400-900°C 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).

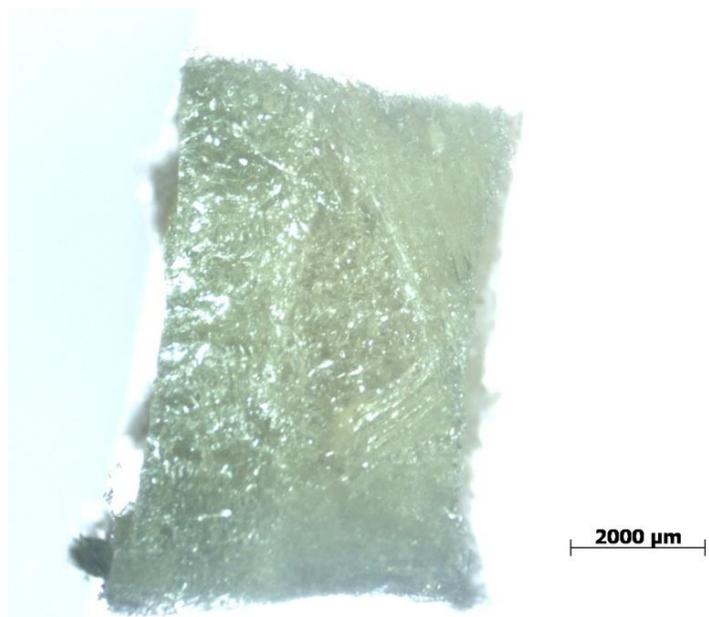
X.3.2 Solvent casting and particulate-leaching

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 CO₂ gas dispersed throughout a polymer mixed with a porogen (e.g. sodium chloride), until saturation. The solubility of CO₂ 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.

References

Abdelaal O, Darwish S (2011) Fabrication of tissue engineering scaffolds using rapid prototyping techniques. *World Academy of Science, Engineering and Technology* 59:577-585

Agarwal S, Wendorff J, Greiner A (2008) Use of electrospinning technique for biomedical applications. *Polymer* 49:5603-5621

Aizenberg J, Weaver J, Thanawala MS, Sundar VC, Morse DE, Fratzl P (2005) Skeleton of *Euplectella* sp.: structural hierarchy from the nanoscale to the macroscale. *Science* 309:275-278

Alvarez Perez MA, Guarino V, Cirillo V, Ambrosio L (2012) In vitro mineralization and bone osteogenesis in poly(epsilon-caprolactone)/gelatin nanofibers. *J Biomed Mater Res A* 100 (11):3008-3019

Azami M, Moosavifar M, Baheiraei N, Moztarzadeh F, Ai J (2012) Preparation of a biomimetic nanocomposite scaffold for bone tissue engineering via mineralization of gelatin hydrogel and study of mineral transformation in simulated body fluid. *J Biomed Mater Res A* 100:1347-1355

Baker R (2000) *Membrane Technology and Applications*. McGraw-Hill, USA

Balasubramanian P, Prabhakaran M, Kai D, Ramakrishna S (2013) Human cardiomyocyte interaction with electrospun fibrinogen/gelatin nanofibers for myocardial regeneration. *J Biomater Sci Polym Ed* 24:1660-1675

Beskardes IG, Demirtas TT, Durukan MD, Gumusderelioglu M (2012) Microwave-assisted fabrication of chitosan-hydroxyapatite superporous hydrogel composites as bone scaffolds. *J Tissue Eng Regen Med*. doi: 10.1002/term.1677

Bhumiratana S, Grayson W, Castaneda A, Rockwood D, Gil E, Kaplan D, Vunjak-Novakovic G (2011) Nucleation and growth of mineralized bone matrix on silk-hydroxyapatite composite scaffolds. *Biomater* 32:2812-2820

Binan L, Tendey C, De Crescenzo G, El Ayoubi R, Ajji A, Jolicoeur M (2013) Differentiation of neuronal stem cells into motor neurons using electrospun poly-L-lactic acid/gelatin scaffold. *Biomaterials*:S0142-9612

Blasier RD, Bucholz R, Cole WG, Wohnson L, Makela E (1997) Bioresorbable implants: applications in orthopaedic surgery. *Instr Course Lec AAOS* 46:531-546

Bock N, Woodruff MA, Steck R, Huttmacher DW, Farrugia BL, Dargaville TR (2013) Composites for Delivery of Therapeutics: Combining Melt Electrospun Scaffolds with Loaded Electrospayed Microparticles. *Macromolecular bioscience*. doi: 10.1002/mabi.201300276

Bohner M (2000) Calcium orthophosphates in medicine: from ceramics to calcium phosphate cements. *Injury-International Journal of the Care of the Injured* 31:37-47

Bosworth L, Turner L, Cartmell S (2013) State of the art composites comprising electrospun fibres coupled with hydrogels: a review. *Nanomedicine* 9:322-335

Bryant S, Davis-Arehart K, Luo N, Shoemaker R, Arthur J, Anseth K (2004) Synthesis and characterization of photopolymerized multifunctional hydrogels: water-soluble poly(vinyl alcohol) and chondroitin sulfate macromers for chondrocyte encapsulation. *Macromolecules* 37:6726-6733

Bucholtz R, Henry S, Henley M (1994) Fixation with bioabsorbable screws for the treatment of fractures of the ankle. *J Bone Joint Surg* 76:319-324

Cao Y, Croll T, Lees J, Tuch B, Cooper-White J (2005) Scaffolds, stem cells, and tissue engineering: A potent combination. *Aust J Chem* 58 691-703

Causa F, Netti P, Ambrosio L, Ciapetti G, Baldini N, Pagani S, Martini D, Giunti A (2006) Poly-ε-caprolactone/hydroxyapatite composites for bone regeneration: in vitro characterization and human osteoblast response. *J Biomed Mater Res A* 76:151-162

Chang W, Mu X, Zhu X, Ma G, Li C, Xu F, Nie J (2013) Biomimetic composite scaffolds based mineralization of hydroxyapatite on electrospun calcium-containing poly(vinyl alcohol) nanofibers. *Materials science & engineering C, Materials for biological applications* 33 (7):4369-4376

Cheng Q, L.-P. Lee B, Komvopoulos K, Li S (2013) Engineering the Microstructure of Electrospun Fibrous Scaffolds by Microtopography. *Biomacromolecules* 14:1349–1360

Chung H, Park T (2009) Self-assembled and nanostructured hydrogels for drug delivery and tissue engineering. *Nano Today* 4:429-437

Columbus S, Krishnan L, Kalliyana K (2013) Relating pore size variation of poly (ε-caprolactone) scaffolds to molecular weight of porogen and evaluation of scaffold properties after degradation. *J Biomed Mater Res B Appl Biomater*. doi: 10.1002/jbm.b.33060

Daculsi G, Laboux O, Malard O, Weiss P (2003) Current state of the art of biphasic calcium phosphate bioceramics. *Journal of Materials Science-Materials in Medicine* 14 (3):195-200

De Nardo L, Bertoldi S, Cigada A, Tanzi M, Haugen H, Farè S (2012) Preparation and characterization of shape memory polymer scaffolds via solvent casting/particulate leaching. *J Appl Biomater Funct Mater* 10:119-126

Dehghani F, Annabi N (2011) Engineering porous scaffolds using gas-based techniques. *Current Opinion in Biotechnology* 22:661-666

Engel E, Michiardi A, Navarro M, Lacroix D, Planell J (2008) Nanotechnology in regenerative medicine: The materials side. *Trends in Biotechnology* 26:39-47

Fabbri P, Bondioli F, Messori M, Bartoli C, Dinucci D, Chiellini F (2010) Porous scaffolds of polycaprolactone reinforced with in situ generated hydroxyapatite for bone tissue engineering. *J Mater Sci Mater Med* 21:343-351

Finkemeier CG (2002) Bone-grafting and bone-graft substitutes. *Journal of Bone and Joint Surgery-American* 84:454-464

Floren M, Spilimbergo S, Motta A, Migliaresi C (2011) Porous poly(D,L-lactic acid) foams with tunable structure and mechanical anisotropy prepared by supercritical carbon dioxide. *J Biomed Mater Res B Appl Biomater* 99 (2):338-349

Gardel LS, Serra LA, Reis RL, Gomes ME (2013) Use of Perfusion Bioreactors and Large Animal Models for Long Bone Tissue Engineering. *Tissue engineering Part B, Reviews*. doi:10.1089/ten.TEB.2013.0010

Gaspar D, Gomide V, Monteiro F (2012) The role of perfusion bioreactors in bone tissue engineering. *Biomater* 2 (4):167-175

Gomes M, Azevedo H, Moreira A, Ella V, Kellomaki M, Reis R (2008) Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: structure, mechanical properties and degradation behaviour. *Journal of Tissue Engineering and Regenerative Medicine* 2 (5):243-252

Hiemstra C, van der Aa L, Zhong Z, Kijkstra P, Jan F (2007) Rapidly in situ-forming degradable hydrogels form dextran thiols through Michael addition. *Biomacromolecules* 8:1548-1556

Hoelzle D, Alleyne A, Johnson A (2008) Micro-robotic deposition guidelines by a design of experiments approach to maximize fabrication reliability for the bone scaffold application. *Acta Biomaterialia* 4:897

Hoffman A (2002) Hydrogels for biomedical applications. *Adv Drug Deliver Rev* 43:3-12

Hong J, Madhally S (2011) Next generation of electrosprayed fibers for tissue regeneration. *Tissue Eng, Part B: Rev* 17:125-142

Hottot A, Vessot S, Andrieu J (2004) A direct characterization method of the ice morphology. Relationship between mean crystals size and primary drying times of freeze-drying processes. *Dry Technol* 22:2009-2021

Hou QP, Grijpma DW, Feijen J (2003) Porous polymeric structures for tissue engineering prepared by a coagulation, compression moulding and salt leaching technique. *Biomaterials* 24:1937-1947

Hunter KT, Ma T (2013) In vitro evaluation of hydroxyapatite-chitosan-gelatin composite membrane in guided tissue regeneration. *J Biomed Mater Res A* 101 (4):1016-1025

Jia L, Prabhakaran MP, Qin X, Ramakrishna S (2013) Stem cell differentiation on electrospun nanofibrous substrates for vascular tissue engineering. *Materials science & engineering C, Materials for biological applications* 33 (8):4640-4650

Kim HJ, Kim U-J, Kim HS, Li C, Wada M, Leisk GG, Kaplan DL (2008) Bone tissue engineering with premineralized silk scaffolds. *Bone* 42 (6):1226-1234

Kokubo T, Takadama H (2006) How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* 27:2907-2915

Kolluru P, Lipner J, Liu W, Xia Y, Thomopoulos S, Genin G, Chasiotis I (2013) Strong and tough mineralized PLGA nanofibers for tendon-to-bone scaffolds. *Acta Biomater*:S1742-7061

Kotela I, Podporska J, Soltysiak E, Konsztowicz J, Blazewicz M (2009) Polymer nanocomposites for bone tissue substitutes. *Ceramics International* 35 2475-2480

Kretlow J, Klouda L, Mikos A (2007) Injectable matrices and scaffolds for drug delivery in tissue engineering. *Adv Drug Deliver Rev* 59:263-273

Langer R, Tirrell D (2004) Designing materials for biology and medicine. *Nature* 428:487-492

Li JL, Cai YL, Guo YL, Fuh JY, Sun J, Hong GS, Lam RN, Wong YS, Wang W, Tay BY, Thian ES (2013) Fabrication of three-dimensional porous scaffolds with controlled filament orientation and large pore size via an improved E-jetting technique. *J Biomed Mater Res B Appl Biomater*. doi:10.1002/jbm.b.33043

Li L, Li G, Jiang J, Liu X, Luo L, Nan K (2012) Electrospun fibrous scaffold of hydroxyapatite/poly (epsilon-caprolactone) for bone regeneration. *J Mater Sci Mater Med* 23 (2):547-554

Liapis AI, Pikal MJ, Bruttini R (1996) Research and development needs and opportunities in freeze drying. *Dry Technol* 14:1265-1300

Lima M, Pirraco R, Sousa R, Neves N, Marques A, Bhattacharya M, Correlo V, Reis R (2013) Bottom-up approach to construct microfabricated multi-layer scaffolds for bone tissue engineering. *Biomed Microdevices*

Liu C, Abedian R, Meister R, Haasper C, Hurschler C, Krettek C, von Lewinski G, Jagodzinski M (2012) Influence of perfusion and compression on the proliferation and differentiation of bone mesenchymal stromal cells seeded on polyurethane scaffolds. *Biomaterials* 33 (4):1052-1064

Liu S, Tay R, Khan M, Ee P, Hedrick J, Yang Y (2010) Synthetic hydrogels for controlled stem cell differentiation. *Soft Matter* 6:67-81

Lu X, Zheng B, Tang X, Zhao L, Lu J, Zhang Z, Zhang J, Cui W (2011) In vitro biomechanical and biocompatible evaluation of natural hydroxyapatite/chitosan composite for bone repair. *Journal of applied biomaterials & biomechanics* : JABB 9 (1):11-18

Lv Q, Nair L, Laurencin CT (2009) Fabrication, characterization, and in vitro evaluation of poly(lactic acid glycolic acid)/nano-hydroxyapatite composite microsphere-based scaffolds for bone tissue engineering in rotating bioreactors. *J Biomed Mater Res A* 91 (3):679-691

Malafaya P, Reis R (2003) Porous bioactive composites from marine origin based in chitosan and hydroxyapatite particles. *Key Eng Mater* 240-242:39-42

Manda-Guiba G, Oliveira M, Mano J, Marques A, Oliveira J, Correlo V, Reis R (2012) Gellan gum - hydroxyapatite composite hydrogels for bone tissue engineering 6:15

Mandoli C, Mecheri B, Forte G, Pagliari F, Pagliari S, Carotenuto F, Fiaccavento R, Rinaldi A, Di Nardo P, Licoccia S, Traversa E (2010) Thick soft tissue reconstruction on highly perfusive biodegradable scaffolds. *Macromol Biosci* 10:127-138

Mano JF, Silva GA, Azevedo HS, Malafaya PB, Sousa RA, Silva SS, Boesel LF, Oliveira JM, Santos TC, Marques AP, Neves NM, Reis RL (2007) Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. *J R Soc Interface* 4:999-1030

Miranda P, Saiz E, Gryn K, Tomsia A (2006) Sintering and robocasting of beta-tricalcium phosphate scaffolds for orthopaedic applications. *Acta Biomaterialia* 2:457-466

Moore M, Jabbari E, Ritman E, Lu L, Currier B, Windebank A, Yaszemski M (2004) Quantitative analysis of interconnectivity of porous biodegradable scaffolds with micro-computed tomography. *J Biomed Mater Res Part A* 71:258-267

Nam YS, Yoon JJ, Park TG (2000) A novel fabrication method of macroporous biodegradable polymer scaffolds using gas foaming salt as a porogen additive. *J Biomed Mater Res* 53 (1):1-7

Ng R, Zang R, Yang K, Liu N, Yang S (2012) Three-dimensional fibrous scaffolds with microstructures and nanotextures for tissue engineering. *RSC Advances* 2:10110-10124

Nguyen T, West J (2002) Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials* 23:4307-4314

Ni P, Fu S, Fan M, Guo G, Shi S, Peng J, Luo F, Qian Z (2011) Preparation of poly(ethylene glycol)/polylactide hybrid fibrous scaffolds for bone tissue engineering. *Int J Nanomedicine* 6:3065-3075

Oliveira A, Costa S, Sousa R, Reis R (2009a) Nucleation and growth of biomimetic apatite layers on 3D plotted biodegradable polymeric scaffolds: Effect of static and dynamic coating conditions. *Acta Biomaterialia* 5:1626-1638

Oliveira A, Sampaio S, Sousa R, Reis R (2006a) Controlled mineralization of nature-inspired silk fibroin/hydroxyapatite hybrid bioactive scaffolds for bone tissue engineering applications Paper presented at the 20th European Conference on Biomaterial, Nantes, France., 27 September-1 October

Oliveira A, Sousa E, Silva N, Sousa N, Salgado A, Reis R (2012) Peripheral mineralization of a 3D biodegradable tubular construct as a way to enhance guidance stabilization in spinal cord injury regeneration. *J Mater Sci: Mater Med* 23:2821-2830

Oliveira AL, Malafaya PB, Costa SA, Sousa RA, Reis RL (2007) Micro-computed tomography (micro-CT) as a potential tool to assess the effect of dynamic coating routes on the formation of biomimetic apatite layers on 3D-plotted biodegradable polymeric scaffolds. *J Mater Sci Mater Med* 18 (2):211-223

Oliveira J, Martins L, Picciochi R, Malafaya P, Sousa R, Neves N, Mano J, Reis R (2010) Gellan gum: a new biomaterial for cartilage tissue engineering applications. *J Biomed Mater Res A* 93:852

Oliveira J, Silva S, Malafaya P, Rodrigues M, Kotobuki N, Hirose M, Gomes M, Mano J, Ohgushi H, Reis R (2009b) Macroporous hydroxyapatite scaffolds for bone tissue engineering applications: Physicochemical characterization and assessment of rat bone marrow stromal cell viability. *J Biomed Mater Res A* 91:175-186

Oliveira J, Sousa R, Kotobuki N, Tadokoro M, Hirose M, Mano J, Reis R, Ohgushi H (2008) The Osteogenic Differentiation of Rat Bone Marrow Stromal Cells Cultured With Dexamethasone-loaded Carboxymethylchitosan/Poly(amidoamine) Dendrimer Nanoparticles. *Biomaterials* 30 (5):804-813

Oliveira J, Sousa R, Malafaya P, Silva S, Hirose M, Ohgushi H, Mano J, Reis R (2011) In vivo study of dendron-like nanoparticles for stem cells tune-up: From nano to tissues. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 7:914-924

Oliveira JM, Costa SA, Leonor IB, Malafaya PB, Mano JF, Reis RL (2009c) Novel hydroxyapatite/carboxymethylchitosan composite scaffolds prepared through an innovative "autocatalytic" electroless coprecipitation route. *J Biomed Mater Res A* 88 (2):470-480

Oliveira JM, Rodrigues M, Silva SS, Malafaya PB, Gomes ME, Viegas CA, Dias IR, Azevedo JT, Mano JF, Reis RL (2006b) Novel hydroxyapatite/chitosan bilayered scaffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells. *Biomater* 27:6123-6137

Oliveira JT, Reis RL (2011) Polysaccharide-based materials for cartilage tissue engineering applications. *J Tissue Eng Regen Med* 5:421-436

Pascu EI, Stokes J, McGuinness GB (2013) Electrospun composites of PHBV, silk fibroin and nano-hydroxyapatite for bone tissue engineering. *Materials science & engineering C, Materials for biological applications* 33 (8):4905-4916

Pereira D, Canadas R, Silva-Correia J, Marques A, Reis R, Oliveira J (2014) Gellan gum-based Hydrogel Bilayered Scaffolds for Osteochondral Tissue Engineering. *Key Engineering Materials* 587:255-260

Pereira D, Silva-Correia J, Caridade S, Oliveira J, Sousa R, Salgado A, Oliveira J, Mano J, Sousa N, Reis R (2011) Development of gellan gum-based microparticles/hydrogel matrices for application in the intervertebral disc regeneration. *Tissue Eng Part C* 17:961

Popescu LM, Piticescu RM, Antonelli A, Rusti CF, Carboni E, Sfara C, Magnani M, Badilita V, Vasile E, Trusca R, Buruiana T (2013) Recent advances in synthesis, characterization of hydroxyapatite/polyurethane composites and study of their biocompatible properties. *J Mater Sci Mater Med* 24 (11):2491-2503

Rada T, Carvalho P, Santos T, Castro A, Reis R, Gomes M (2013) Chondrogenic Potential of Two hASCs Subpopulations Loaded onto Gellan Gum Hydrogel Evaluated in a Nude Mice Model. *Curr Stem Cell Res Ther* 8:357-364

Rumian L, Wojak I, Scharnweber D, Pamula E (2013) Resorbable scaffolds modified with collagen type I or hydroxyapatite: in vitro studies on human mesenchymal stem cells. *Acta Bioeng Biomech* 15:61-67

Salerno A, Guarnieri D, Iannone M, Zeppetelli S, Netti PA (2010) Effect of micro- and macroporosity of bone tissue three-dimensional-poly(epsilon-caprolactone) scaffold on human mesenchymal stem cells invasion, proliferation, and differentiation in vitro. *Tissue Eng Part A* 16 (8):2661-2673

Shin H, Jo S, Mikos A (2003) Biomimetic materials for tissue engineering. *Biomaterials* 24: 4353-4364

Silva-Correia J, Oliveira J, Caridade S, Oliveira J, Sousa R, Mano J, Reis R (2011) Gellan gum-based hydrogels for intervertebral disc tissue-engineering applications. *J Tissue Eng Regen Med* 5:e97-e107

Silva-Correia J, Zavan B, Vindigni V, Silva T, Oliveira J, Abatangelo G, Reis R (2013) Biocompatibility Evaluation of Ionic- and Photo-Crosslinked Methacrylated Gellan Gum Hydrogels: In Vitro and In Vivo Study. *Adv Healthcare Mater* 2:568-575

Silva TH, Alves A, Ferreira BM, Oliveira JM, Reys LL, Ferreira RJ, Sousa RA, Silva SS, Mano JF, Reis RL (2012) Materials of marine origin: a review on polymers and ceramics of biomedical interest. *Int Mater Reviews*. DOI 10.1179/1743280412Y.0000000002

Slaughter B, Khurshid S, Fisher O, Khademhosseini A, Peppas N (2009) Hydrogels in Regenerative Medicine. *Adv Mater* 21:3307-3329

Smith IO, Liu XH, Smith L, Ma PX (2009) Nanostructured polymer scaffolds for tissue engineering and regenerative medicine. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* 1:226-236

Sprio S, Ruffini A, Valentini F, D'Alessandro T, Sandri M, Panseri S, Tampieri A (2011) Biomimesis and biomorphic transformations: New concepts applied to bone regeneration. *Journal of Biotechnology* 156 (4):347-355

Stankus J, Freytes D, Badylak S, Wagner W (2008) Hybrid Nanofibrous Scaffolds from Electrospinning of a Synthetic Elastomer and Urinary Bladder Matrix *Journal of Biomaterials Science Polymer Ed* 19:635-652

Stuart A, Gonzenbach U, Tervoort E, Gauckler L (2006) Processing Routes to Macroporous Ceramics: A Review. *J Am Ceram Soc* 89:1771-1789

Suh S, Shin J, Kim J, Beak C, Kim D, Kim H, Jeon S, Choo I (2002) Effect of different particles on cell proliferation in polymer scaffolds using a solvent-casting and particulate leaching technique. *ASAIO J* 48:460-464

Tan H, Marra K (2010) Injectable, biodegradable hydrogels for tissue engineering applications. *Materials* 3:1746-1767

Thadavirul N, Pavasant P, Supaphol P (2013) Development of polycaprolactone porous scaffolds by combining solvent casting, particulate leaching, and polymer leaching techniques for bone tissue engineering. *Journal of Biomedical Materials Research: Part A Accepted Article*, doi: 10.1002/jbm.a.35010

Thomas V, Zhang X, Vohra Y (2009) A biomimetic tubular scaffold with spatially designed nanofibers of protein/PDS bio-blends. *Biotechnol Bioeng* 104:1025-1033

Tibbitt M, Anseth K (2009) Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng* 103:655-663

Van Vlierberghe S, Dubrue P, Schacht E (2011) Biopolymer-Based Hydrogels As Scaffolds for Tissue Engineering Applications: A Review. *Biomacromolecules* 12:1387-1408

Van Vlierberghe S, Fritzing B, Martins J, Dubrue P (2010) Hydrogel network formation revised: high-resolution magic angle spinning nuclear magnetic resonance as a powerful tool for measuring absolute hydrogel cross-link efficiencies. *Appl Spectrosc* 64:1176-1180

vandeWitte P, Dijkstra PJ, vandenBerg JWA, Feijen J (1996) Phase separation processes in polymer solutions in relation to membrane formation. *J Memb Sci* 117:1-31

- Vatankhah E, Semnani D, Prabhakaran MP, Tadayon M, Razavi S, Ramakrishna S (2013) Artificial neural network for modeling the elastic modulus of electrospun polycaprolactone/gelatin scaffolds. *Acta Biomater.* doi:10.1016/j.actbio.2013.09.015
- Venugopal J, Low S, Choon A, Kumar A, Ramakrishna S (2008) Electrospun modified nanofibrous scaffolds for the mineralization of osteoblast cells. *J Biomed Mater Res* 85:408e417
- Wang C, Stewart R, Kopecek J (1999) Hybrid hydrogels assembled from synthetic polymers and coiled-coil protein domains. *Nature* 397:417-420
- Wang E, Lee SH, Lee SW (2011) Elastin-like polypeptide based hydroxyapatite bionanocomposites. *Biomacromolecules* 12 (3):672-680
- Wang F, Zhang Y, Zhou H, Guo Y, Su X (2013) Evaluation of in vitro and in vivo osteogenic differentiation of nano-hydroxyapatite/chitosan/poly(lactide-co-glycolide) scaffolds with human umbilical cord mesenchymal stem cells. *J Biomed Mater Res A* doi: 10.1002/jbma.a.34747
- Wang TW, Wu HC, Wang HY, Lin FH, Sun JS (2009) Regulation of adult human mesenchymal stem cells into osteogenic and chondrogenic lineages by different bioreactor systems. *Journal of Biomedical Materials Research - Part A* 88 (4):935-946
- Wei J, Heo SJ, Liu C, Kim DH, Kim SE, Hyun YT, Shin JW, Shin JW (2009) Preparation and characterization of bioactive calcium silicate and poly(epsilon-caprolactone) nanocomposite for bone tissue regeneration. *J Biomed Mater Res A* 90 (3):702-712
- Yan L-P, Correia J, Correia C, Caridade S, Fernandes E, Sousa R, Mano JF, Oliveira J, Oliveira A, Reis RL (2013) Bioactive macro/micro porous silk fibroin/nano-sized calcium phosphate scaffolds with potential for bone-tissue-engineering applications. *Nanomedicine* 8:359-378
- Yang P, Huang X, Wang C, Dang X, Wang K (2013) Repair of bone defects using a new biomimetic construction fabricated by adipose-derived stem cells, collagen I, and porous beta-tricalcium phosphate scaffolds. *Exp Biol Med*
- Yeatts AB, Both SK, Yang W, Alghamdi HS, Yang F, Fisher JP, Jansen JA (2013) In Vivo Bone Regeneration Using Tubular Perfusion System Bioreactor Cultured Nanofibrous Scaffolds. *Tissue Eng Part A.* doi:10.1089/ten.TEA.2013.0168
- Yeatts AB, Fisher JP (2011) Bone tissue engineering bioreactors: dynamic culture and the influence of shear stress. *Bone* 48 (2):171-181
- Yeatts AB, Geibel EM, Fears FF, Fisher JP (2012) Human mesenchymal stem cell position within scaffolds influences cell fate during dynamic culture. *Biotechnol Bioeng* 109 (9):2381-2391
- Yeong W, Chua C, Leong K, Chandrasekaran M (2004) Rapid prototyping in tissue engineering: challenges and potential. *Trends Biotechnol* 22:643-652

Zhang R, Ma P (2001) Processing of polymer scaffolds: phase separation. In: Atala AaL, R. (ed) Methods of Tissue Engineering, vol 263. Academic Press, San Diego

Zhang ZY, Teoh SH, Chong WS, Foo TT, Chng YC, Choolani M, Chan J (2009) A biaxial rotating bioreactor for the culture of fetal mesenchymal stem cells for bone tissue engineering. Biomaterials 30 (14):2694-2704

Zhou C, Shi Q, Guo W, Terrell L, Qureshi AT, Hayes DJ, Wu Q (2013) Electrospun bio-nanocomposite scaffolds for bone tissue engineering by cellulose nanocrystals reinforcing maleic anhydride grafted PLA. ACS applied materials & interfaces 5 (9):3847-3854

Zhu J, Marchant R (2011) Design properties of hydrogel tissue-engineering scaffolds. Expert Rev Med Devices 8:607-626

Zreiqat H (2013) Mimicking Bone Microenvironment for Directing Adipose Tissue-Derived Mesenchymal Stem Cells into Osteogenic Differentiation. Methods Mol Biol. Doi 10.1007/7651_2013_38