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REVIEW

Plug and play: combining materials and technologies to improve bone regenerative strategies

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Abstract

Despite recent advances in the development of biomaterials intended to replace natural bone grafts for the regeneration of large, clinically relevant defects, most synthetic solutions that are currently applied in the clinic are still inferior to natural bone grafts with regard to regenerative potential and are limited to non-weight-bearing applications. From a materials science perspective, we always face the conundrum of the preservation of bioactivity of calcium phosphate ceramics in spite of better mechanical and handling properties and processability of polymers. Composites have long been investigated as a method to marry these critical properties for the successful regeneration of bone and, indeed, have shown a significant improvement when used in combination with cells or growth factors. However, when looking at this approach from a clinical and regulatory perspective, the use of cells or biologicals prolongs the path of new treatments from the bench to the bedside. Applying 'smart' synthetic materials alone poses the fascinating challenge of instructing tissue regeneration *in situ*, thereby tremendously facilitating clinical translation. In the journey to make this possible, and with the aim of adding up the advantages of different biomaterials, combinations of fabrication technologies arise as a new strategy for generating instructive three-dimensional (3D) constructs for bone regeneration. Here we provide a review of recent technologies and approaches to create such constructs and give our perspective on how combinations of technologies and materials can help in obtaining more functional bone regeneration. Copyright © 2013 John Wiley & Sons, Ltd.

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1. Introduction

Bone is probably one of the best examples of tissues for which an increasing need exists for regenerative strategies, as a consequence of population ageing. To realize the extent and prevalence of bone-related diseases and disorders, it is interesting to note that 2000–2010 was appointed as a Bone and Joint Decade by the World Health Organization (Cambron and King, 2006). Musculoskeletal conditions are the most common causes of severe long-term pain and physical disability, and account for half of all chronic conditions in people aged over 50 years in developed countries. In 1998, in the USA, about 220 000 cases of spinal fusion requiring a bone graft were performed. Each year, approximately 170 000 fractures do not heal and are diagnosed as 'non-unions', requiring some form of bone graft substitute (Chen *et al*., 2003). While autologous bone grafts are still considered the best solution when it comes to treatment of large, critical-sized bone defects, the limited availability is becoming an increasingly important problem as the need for regenerative strategies expands, in addition to other concerns, including donor site morbidity, need for an additional surgery, etc. Other natural bone grafts are widely used too, but their performance is often inferior to that of autografts as a consequence of treatments to avoid disease transmission to and immunogenic response by the recipients.

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As an alternative to natural bone grafts, various tissue-engineering approaches have been developed in the past 25 years in an attempt to mimic the role of natural bone grafts as closely as possible. Tissue-engineering approaches for bone repair and regeneration are based on stimulating bone formation through the use of growth factors and/or cells in combination with a biomaterial scaffold. Bone morphogenetic proteins (BMPs), for example, were discovered and defined as compounds present in demineralized bone matrix (DBM) that act as sole inducers of *de novo* bone formation (Urist, 1965). Owing to their osteoinductive potential, two BMP family members have found their way to the clinic, where they are used for enhancing non-unions of long bones, spinal fusion, craniomaxillofacial disease and for periodontal and dental indications, as reviewed elsewhere (Kirker-Head, 2000). The advances in recombinant DNA technology have also allowed for the availability of BMPs readily and in large quantities. BMP therapy is, however, based on the application of supraphysiological concentrations and is therefore accompanied by the risk of, for example, heterotopic bone formation (Haidar *et al*., 2009). FDA approval for the application of BMP-2 and BMP-7, therefore, comes with restrictions. In the conventional tissue-engineering cell-based approach, cells are obtained from the patient, expanded on two-dimensional (2D) surfaces such as plates or flasks, seeded on a three-dimensional (3D) scaffold, to finally be implanted into the patient for repair and regeneration. While somewhat contradictory results exist regarding the (pre)clinical successes of cell-based tissue-engineering constructs (Meijer *et al*., 2007), no products have yet reached the clinic. One of the reasons for this may also be the high costs associated with the production and storage of cell-based tissue-engineered constructs.

While a number of synthetic materials had been used for treating bone defects long before tissue-engineered constructs were developed, they have never really been considered a true alternative to natural bone grafts. An important reason is that the majority of these bone graft substitutes were inferior to their natural counterparts in terms of biological response. While most synthetic bone graft substitutes have been shown to be biocompatible and osteoconductive, they are generally considered non-osteoinductive, which makes them inferior in the treatment of large, critical-sized defects as compared to natural bone grafts and constructs containing osteoinductive growth factors. Osteoinduction is the process of differentiation of undifferentiated cells into the osteogenic lineage (Friedenstein, 1968), followed by bone formation, and is believed to be the key element for closing large, critical-sized bone defects. Nevertheless, the idea of using synthetic biomaterials for the treatment of large bone defects has been revisited in the past decade, as they are relatively inexpensive and available in large quantities off-the-shelf. Much effort has also been put into improving their biological performance and a number of groups have reported biomaterials, generally calcium phosphate ceramics, with intrinsic osteoinductivity (Barradas *et al*., 2011). Intrinsically osteoinductive materials are an example of 'instructive' or 'smart' synthetic materials, owing to their ability, directly or indirectly, to trigger osteogenic differentiation of non-bony cells in the heterotopic environment, followed by *de novo* bone formation. While indications and hypotheses exist identifying surface features, including microporosity and grain size as well as dissolution/reprecipitation events occurring on the surface, as properties responsible for the osteoinductive potential of synthetic biomaterials, the exact biological mechanism behind osteoinduction is still incompletely understood (Barradas *et al*., 2011). An important reason is that the level of control over the properties of osteoinductive calcium phosphate ceramics is limited and dependent on processing parameters. Efforts to design 'smart' biomaterials while retaining their synthetic character are growing, as is illustrated by elegant work on control of cell fate by tailoring surface micro- and nanopatterning (Dalby *et al*., 2007; Kilian *et al*., 2010; McBeath *et al*., 2004; McMurray *et al*., 2011; Unadkat *et al*., 2011), mechanical properties and chemical surface functionalization of the materials (Huebsch *et al*., 2010; Kshitiz *et al*., 2012; Lutolf and Blau, 2009; Villa-Diaz *et al*., 2010). However, most methods to design and engineer surface properties of solid materials are solely applicable to 2D substrates and limited to polymers. Functionalization of hydrogels can be performed within an intrinsic 3D environment (Benoit *et al*., 2008; DeForest *et al*., 2009; Kobel and Lutolf, 2012; Luo and Shoichet, 2004). However, successful regeneration of large bone defects requires functional 3D materials, ideally able to withstand mechanical loading. Therefore, a challenging step remains to be taken to optimally employ advances in surface control to significantly improve the biological performance of synthetic bone graft substitutes. Indeed, while desired biological performance is a prerequisite for the success of a material as a bone graft substitute, other requirements also need to be met. As previously mentioned, mechanical properties are important, particularly when it comes to load-bearing applications. In that respect, not only the type of biomaterial but also its structural properties (e.g. porosity, interconnectivity) when processed to create a 3D scaffold play an important role. Handling properties, such as injectability and ease of surgical manipulation, constitute another issue that needs to be considered in specific applications.

While many excellent reviews exist on different types of biomaterials used in bone-regenerative strategies (Damien and Parsons, 1991; Dinopoulos *et al*., 2012), the focus of the current paper is to review recently undertaken approaches to develop, employ and combine materials and fabrication technologies in order to provide plug-and-play systems able to meet the specific requirements of an application in both basic research and clinical use.

2. Building 3D constructs for bone regeneration

Calcium phosphate ceramics (Yuan *et al*., 2010), such as hydroxyapatite (HA) (Deville *et al*., 2006; Yoshikawa *et al*., 2009), tricalcium phosphate (TCP) (Dong *et al*., 2002;

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Fu *et al*., 2000; Niedhart *et al*., 2001) and biphasic calcium phosphate (BCP), consisting of HA and TCP (Legeros *et al*., 2003), have a long history of use in bone-regenerative strategies, owing to their similarity in chemical composition to bone mineral and related bioactivity in terms of osteoconductivity and, sometimes, even osteoinductivity (Barradas *et al*., 2011; Damien and Parsons, 1991). An important disadvantage of most calcium phosphate ceramics is their intrinsic brittleness (Zimmermann and Moghaddam, 2011), limiting their use to non-load bearing or supported applications. With the rationale that in natural bone the organic component (predominantly collagen) is responsible for excellent mechanical properties, it is not surprising that many strategies focus on a combination of calcium phosphate ceramics and natural or synthetic polymers in an attempt to develop bone graft substitutes with improved mechanical properties, without compromising bioactivity. Combinations of calcium phosphate ceramics with metals that are widely used in orthopaedics, such as titanium and its alloys, is another approach to improve mechanical properties. However, considering the non-degradability of most metals, this possibility is less explored in the context of tissue regeneration. Apart from the intrinsic mechanical properties of different material types, the way they are built into 3D constructs and consequent properties of, for example, the resulting porous structure, will greatly determine the mechanical properties of the resulting cellular solid.

Calcium phosphate ceramics are usually produced as (porous) granules, beads or scaffolds with a defined shape, which allows for implantation into confined defects using conventional surgical procedures. With an increasing need to perform minimally invasive surgical procedures, it is necessary to improve the handling properties of ceramics, making them mouldable or injectable, which can also be achieved by combining ceramic particles with polymers that enable the desired handling. In the next section we discuss scaffolding techniques that are used to build 3D constructs for bone regeneration in an attempt to control mechanical and handling properties and biological performance.

3. Conventional scaffold fabrication technologies

First-generation polymeric scaffolds used in regenerative strategies were fabricated using conventional methods such as solvent casting (Mikos *et al*., 1993), gas foaming (Mooney *et al*., 1996), freeze drying (Schoof *et al*., 2001) and particulate leaching (Claase *et al*., 2003). For ceramics and metals powder metallurgical processing technologies were used, including (polymer) replica techniques (Colombo, 2006; Luyten *et al*., 2009; Ramay and Zhang, 2003), direct foaming of a liquid slurry and burning out of fugitive porogens (Chen *et al*., 2006; Colombo, 2006; Padilla *et al*., 2005), followed by calcination and sintering. Although these techniques are very useful for fabricating scaffolds, they suffer from some drawbacks. While it is possible to control pore size and shape by altering processing conditions, fabrication of fully interconnected scaffolds remains a challenge. Low interconnectivity leads to tortuous paths in the scaffolds, which influence infiltration of the scaffold by cells and tissue and their survival. Combinations of the conventional technologies with template porogens of highly controlled dimensions at the micro- and nanoscale have improved pore interconnectivity. Inverted colloidal crystal (ICC), in particular, was used to fabricate poly(lactic-co-glygolic acid) (PLGA) 3D scaffolds with an interconnected pore architecture mimicking that of cancellous bone (Cuddihy and Kotov, 2008). These scaffolds were also shown to be useful in setting the first steps to recreate an *in vitro* model of bone marrow (Nichols *et al*., 2009) and, more recently, the combination of ICC with layer-by-layer approach added to versatility in the fabrication of scaffolds with enhanced (bio)functionalities (Andres *et al*., 2012). However, pore tortuosity and strut fragility are critical characteristics that need to be improved when aiming to build scaffolds able to regenerate vascularized bone tissue and with mechanical properties able to sustain dynamic loading. In order to overcome the limitations associated with conventional scaffold fabrication technologies, rapid prototyping (RP) techniques have been employed.

4. Rapid prototyping

In the past 25–30 years, several RP or solid freeform fabrication (SFF) systems were developed and commercialized for the manufacture of prototypes used in various industries, such as the aerospace, automotive and consumer industries, in electrical and electronic products and in biomedical applications (Chua *et al*., 2010). As the name suggests, these techniques fabricate parts or prototypes without the use of moulds. Parts are built layer-by-layer by additive manufacturing through computer-aided design/computer-aided manufacturing (CAD/CAM), which is opposite to the usual practice of removing materials during conventional fabrication processes. Additionally, RP can be used to fabricate controlled structures that can later be used as negative replicas or sacrificial moulds to fabricate scaffolds. In the last decade, RP technologies have been widely used to fabricate 3D scaffolds for a number of applications, due to various advantages. RP technologies offer more control compared to conventional technologies and can reproducibly fabricate parts. Since the fabrication is performed layer by layer, it is also possible to modify properties of individual layers to obtain complex 3D structures. The possibility of tuning various aspects of scaffold properties, such as porosity, interconnectivity, mechanical strength and degradation, makes RP a powerful tool for scaffold production and allows the fabrication of customized scaffolds with properties that match a specific application (Hollister, 2005; Lin *et al*., 2004). RP technologies can also be integrated with standard medical imaging processes, such as computed tomography (CT) or magnetic resonance imaging

(MRI) to create customized implants for patients (Figure 1), which in some cases has proved to be effective in the reconstruction of complex and large bone segments (Hutmacher *et al.*, 2004; Li *et al.*, 2011).

RP technologies are predominantly used to build polymeric scaffolds; nevertheless, ceramics, metals and various composites have also been processed into 3D constructs for bone regeneration using RP techniques. Based on the type of technology used, RP systems can be classified into extrusion-, laser- and printing-based systems. Figure 2 displays a schematic overview of the different types of RP systems (Hollister, 2005). The use of RP technologies and the control they provide over parameters such as pore size and shape enabled the creation of different scaffolds, in which the mechanical properties could be modulated to match those of a particular tissue to be repaired (Moroni *et al.*, 2006b).

The advantages of extrusion-based systems include the processing of different material types alone or in the same construct, no trapping of unused materials inside the final construct and only temporary heating of raw materials at elevated temperatures, which limits the negative effects of long-term heating, such as thermal degradation. Among extrusion-based systems, fused deposition modelling (FDM) is a very popular technique for fabricating scaffolds. FDM involves the extrusion of the material in a layered way to create scaffolds. While in the past it was only possible to use few non-resorbable polymeric materials, e.g. acrylonitrile butadiene styrene, polyamide, current FDM systems can process polymers, such as poly(ε-caprolactone) (PCL), poly(L-lactic acid) (PLLA) and PLGA, that are widely used in biomedical applications. Extensive work on the fabrication, characterization and testing of 3D polymeric scaffolds from PCL and composites such as PCL/TCP and PCL/HA for bone regeneration and tissue engineering has been performed by Hutmacher and co-workers (Hutmacher *et al.*, 2001; Lam *et al.*, 2009; Schantz *et al.*, 2005; Yefang *et al.*, 2007; Zhou *et al.*, 2007). These authors have shown, for example, that co-culture of endothelial cells and bone marrow-derived fibroblasts on such 3D scaffolds led to enhanced cell differentiation (Choong *et al.*, 2006). Recently, it has been demonstrated that PCL–TCP scaffold, produced using FDM in combination with BMP-7 infusion, resulted in faster regeneration of a critical-sized long bone defect in sheep, with improved mechanical properties and remodelling of

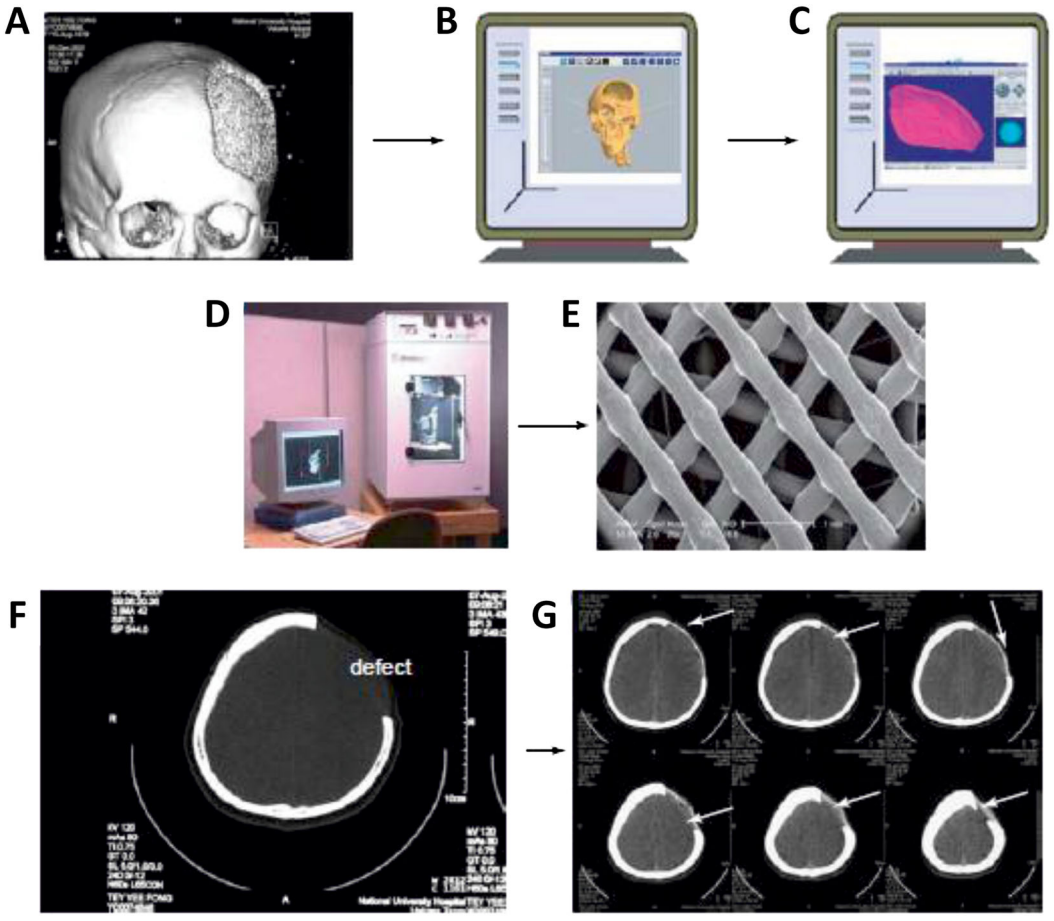

Figure 1. Development of patient-specific bone graft substitutes by combining medical imaging, computational modelling and rapid prototyping (RP). CT scan data of the patient's bone defect (A) are used to generate a computer-based 3D model (B). This model is then imported into RP system software to be 'sliced' into thin horizontal layers, with the tool path specified for each layer (C). The 'sliced' data are used to instruct the RP machine (D) to build a scaffold (E) layer by layer, based on the actual shape of the computer model (C). RP technology produces excellent templates for the treatment of intricate bone defects (A, F). Custom-made constructs (G, see arrows) exactly follow the complex shaped 3D contour of the skull. Reprinted with permission from Hutmacher *et al.* (2004)

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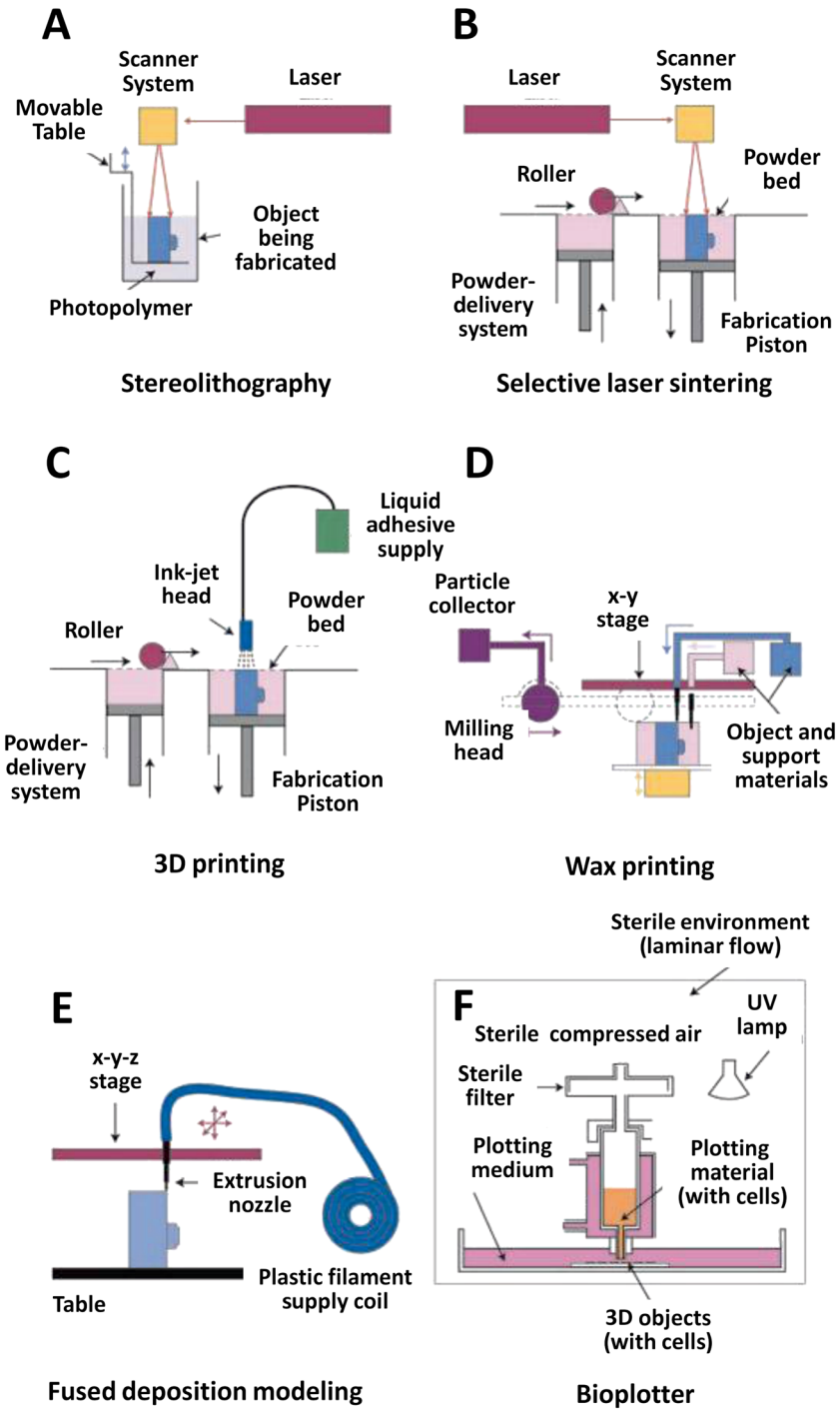

Figure 2. Different solid freeform fabrication systems categorized by processing technique. (A, B) Laser-based processing systems include the stereolithography system, which photopolyermerizes a liquid (A) and the SLS systems, which sinter powdered material (B); in each system, material is swept over a build platform that is lowered for each layer. (C, D) Printing-based systems include 3D printing (C) and a wax printing machine (D); 3DP prints a chemical binder onto a powder bed. The wax-based system prints two types of wax material in sequence. (E, F) Nozzle-based systems: fused deposition modeling prints a thin filament of material that is heated through a nozzle (E). The Bioplotter prints materials that are processed either thermally or chemically (F). Reprinted with permission from Hollister (2005)

the regenerated bone tissue compared to autograft and scaffolds used alone or seeded with bone marrow-derived mesenchymal stromal cells (MSCs) (Reichert *et al.*, 2012). Although the performance of scaffolds alone was inferior to that of scaffolds loaded with the growth factor, the authors suggested that scaffolds contributed to successful bone healing, through their mechanical properties sufficient for weight bearing as well as the capacity to locally deliver BMP-7 necessary for osteoinduction. Furthermore, scaffolds without cells or growth factors were able to provoke considerable bone ingrowth across the defect, which was not observed in empty control defects. 3D fibre deposition (3DF), which is a variant of FDM, has been used for the fabrication of scaffolds for bone and cartilage regeneration.

The 3DF process can be done by extruding molten polymers, hydrogels or pastes in the form of a fibre. In an attempt to control mechanical properties and to improve bioactivity by tailoring macrostructure, scaffolds were fabricated from polymers (Gloria *et al*., 2012; Moroni *et al*., 2008; Sobral *et al*., 2011; Woodfield *et al*., 2004), metals (Li *et al*., 2005a; Li *et al*., 2010), calcium phosphate ceramics (Miranda *et al*., 2006) and polymer–ceramic composites (Nandakumar *et al*., 2013a).

Techniques based on lasers include stereolithography (SLA) and selective laser sintering (SLS). In SLA, UV or visible light lasers are vector-scanned on a bath that contains a photopolymerizable resin. The laser cures the resin at specific areas where it is illuminated and creates a solid layer that attaches to the support platform. This process is repeated layer by layer to create a 3D structure based on a CAD model. Excess resin is washed out and the sample may be cured in a UV oven if needed (Melchels *et al*., 2010b). SLA can achieve resolutions of around 20 μm (other RP techniques are in the range 50–200 μm) and it is relatively easy to remove the support materials used during fabrication, making this technique potentially applicable in trauma surgery for fracture fixation devices, parts of hip- and knee implants and as nerve guidance channels and prostheses (Melchels *et al*., 2010b). Nevertheless, the biggest limitation of SLA is poor availability of photopolymerizable biomaterial resins with the desired properties (Melchels *et al*., 2010b; Yang *et al*., 2002). Therefore, SLA was primarily used for creating 3D models that improved the spatial understanding of the anatomy and physiology and assisted surgeons by reducing the time and risk involved in surgery (Binder *et al*., 2000; Sarment *et al*., 2003). In the last decade biodegradable resins based on poly(propylene fumarate) (PPF) (Cooke *et al*., 2003), trimethylene carbonate (TMC) and ε-caprolactone (CL) (Lee *et al*., 2008; Matsuda and Mizutani, 2002; Matsuda *et al*., 2000), D,L-lactide (DLLA) (Jansen *et al*., 2009; Melchels *et al*., 2009) and photo-curing modified natural polymers (Qiu *et al*., 2009; Schuster *et al*., 2009; Smeds *et al*., 1999; Zimmermann *et al*., 2002) have been synthesized and used to fabricate 3D scaffolds by SLA for biomedical applications. Initial studies with PDLLA SLA scaffolds showed improved cell distribution, while PPF SLA scaffolds were shown to support a more robust and rapid differentiation of rat MSCs into the osteogenic lineage when compared to similar scaffolds fabricated by conventional porogen leaching, which was attributed to enhanced stiffness and improved scaffold permeability (Kim *et al*., 2011; Melchels *et al*., 2010a). An improvement of SLA is two-photon polymerization (2PP), in which a photosensitive resin is polymerized by a laser beam (Ovsianikov and Chichkov, 2012; Ovsianikov *et al*., 2007). The resolution of this RP technology can reach the sub-micron scale. Although new biomaterials with satisfactory biocompatibility have been synthesized and the speed of fabrication has improved, the resins that are readily available for 2PP need to prove their biocompatibility *in vivo*, and the production speed to reach clinically relevant dimensions also needs to be further improved. Therefore, applications in the field of bone regeneration are still too premature with this technology.

SLS works in a manner similar to SLA, with the difference that a powder bed is sintered selectively using a laser. The interaction of the material with the laser causes an increase in the temperature of the material and sintering occurs at temperatures slightly higher than glass transition temperature, which fuses the particles. Subsequent layers are fabricated on top of existing layers and new powder is deposited using a roller. Similarly to SLA, SLS can potentially be used for the production of customized implants for the treatment of large bone defects caused by trauma, tumour removal and similar events. Using SLS, polymers such as PCL, PLLA, poly(ether ether ketone) (PEEK) and poly(hydroxybutyrate-co-hydroxyvalerate) have been combined with ceramics to create composite bioactive scaffolds for bone regeneration, and showed enhanced osteoblast proliferation and differentiation when compared to similar scaffolds without the inclusion of ceramics (Antonov *et al*., 2004; Duan *et al*., 2010; Tan *et al*., 2003; von Wilmowsky *et al*., 2008; Williams *et al*., 2005). PCL SLS scaffolds also showed successful initial tissue formation when seeded with BMP-7-transduced fibroblasts after subcutaneous implantation (Williams *et al*., 2005). Although with SLS it is possible to significantly increase the percentage of ceramic particles ($> 50\%$) into the polymeric phase of composites, the final results are still not at par with what is expected. Recently, Lohfeld *et al*. (2012) showed that the introduction of TCP in PCL SLS scaffolds resulted in lower bone tissue formation in a critical-sized defect *in vivo* compared to PCL, likely due to the mismatch between the PCL and the TCP particle size and to the resulting inferior mechanical properties at high TCP concentrations. High accuracy, good mechanical strength and a broad choice of materials are some of the advantages of SLS, while high processing temperatures, difficulty in removing entrapped materials and the inability to process hydrogels still remain current drawbacks (Melchels *et al*., 2010b; Yang *et al*., 2002). For metals, electrical beam sintering, a method similar to SLS was used to directly build 3D scaffolds (Hollister *et al*., 2005; Mazumder *et al*., 2000).

3D printing (3DP) has the advantage of being able to fabricate 3D structures at ambient temperatures (Sachs *et al*., 1994). Fresh powder is deposited on a stage, onto which a binder solution is subsequently printed by an inkjet head, following a specific CAM pattern. After a layer is complete, the process is continued in a layer-by-layer manner similarly to the previously described RP technologies. Weak bonding between layers and the difficulty of removing entrapped materials that could potentially lead to the incorporation of the binder material in the final scaffold, causing toxicity problems, are some disadvantages of this method (Peltola *et al*., 2008; Yang *et al*., 2002). 3DP was used for fabricating ceramic moulds to cast materials needed for orthopaedic implants (Curodeau *et al*., 2000) and a combination of PLGA, PLA and TCP was used to fabricate scaffold constructs for the repair of articular cartilage (Sherwood *et al*., 2002). PLGA 3DP scaffolds were shown to support osteoconduction when implanted in orthotopic locations in a rabbit model (Ge *et al*., 2009). Although these studies reported promising results for 3DP scaffolds in musculoskeletal applications, in order

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to completely overcome biocompatibility issues posed by using organic solvents, such as chloroform as binders, calcium phosphate-based ceramics have been directly fabricated into scaffolds for bone regeneration using biocompatible or water-soluble polymeric binders that can be removed during sintering at high temperatures (Khalyfa *et al*., 2007; Leukers *et al*., 2005; Seitz *et al*., 2005). 3D printing was also applied to directly deposit calcium and magnesium phosphate cements at ambient temperatures (Gbureck *et al*., 2007; Klammert *et al*., 2010). Such scaffolds were shown to be suitable as drug delivery vehicles (Gbureck *et al*., 2007), as well as for vertical bone augmentation in a calvarial model in rabbits (Torres *et al*., 2011).

Another printing-based technology that is important to mention here, although it always involves cells, is organ printing. Mironov *et al*. (2003) defined organ printing as: 'a rapid prototyping computer-aided 3D printing technology, based on using layer-by-layer deposition of cell and/or cell aggregates into a 3D gel with sequential maturation of the printed construct into perfused and vascularized living tissue or organ'. The process of organ printing can be divided into three steps: (a) preprocessing, dealing with the development of a CAD model for the organ; (b) processing, the actual layer-by-layer printing of cells or aggregates into a 3D structure based on the design; and (c) postprocessing, the perfusion of printed organs and their biomechanical conditioning to both direct and accelerate organ maturation. A few promising approaches have already shown successful fabrication of viable bioprinted osteochondral and vascularized bone constructs (Fedorovich *et al*., 2010, 2012b), where cell-laden hydrogels, alone or in combination with ceramic particles, were used (Fedorovich *et al*., 2012a). Further research should aim at understanding the mechanical stability and bone-forming capacities of these constructs *in vivo* in orthotopic defects in large animal models. While the homogeneous distribution of cells and the creation of a 3D cellular construct are advantageous, the organ-printing method has several limitations, including the choice of scaffold materials that can be used. In the case of solid polymers, most scaffold materials need a strong solvent for dissolution and, hence, printing has been restricted to hydrogels and thermo-reversible polymers that lack rigidity (Ringeisen *et al*., 2006). Furthermore, the amount of hydrogel that can be printed is rather limited. Therefore, new formulations with improved mechanical properties, fast gelation time and versatility to be further functionalized with biological moieties are needed.

5. Electrospinning

Although RP technologies have improved in terms of resolution, it is still impossible to reproduce features in the few micrometres-to-submicron range, which would be relevant to mimic the physical properties of native extracellular matrix (ECM). It is due to this need that electrospinning, a technique originally developed for the field of textiles and filtration of aerosols, has become popular with biomedical researchers. Cooley (1902) and Morton (1902) independently patented the method of electrically dispersing fluids in 1902. Formhals (1934) patented the practical results of producing silk-like threads, using cellulose-based polymer solutions in probably the first instance of producing polymeric threads using electrical fields. In the late 1930s Petryanov-Sokolov used electrostatic fields to produce aerosol filters and the term 'electrospinning' was first introduced in publications in the 1990s (Doshi and Reneker, 1995).

The principle of electrospinning is based on the phenomenon that when a sufficiently high voltage is applied to a liquid droplet, it becomes charged and the electrostatic forces of repulsion counteract the surface tension. As the intensity of the electric field is increased, the hemispherical surface of the drop is stretched to form a conical shape, known as a Taylor cone (Taylor, 1969). Once the strength of the electric field overcomes the surface tension, a continuous stream of liquid jet is ejected from the Taylor cone. The charged nature of the jet enables the control of the trajectory using an electric field. During its flight, the jet dries and is collected as non-woven fibres on a collector. Due to the nature of the process, several factors affect the outcome of the spinning process. Doshi and Reneker (1995) classified these properties as solution properties, controlled variables and ambient conditions. Solution properties include viscosity, conductivity, surface tension, molecular weight and dielectric constant, whereas flow rate, applied electric field, air gap between needle and collector, collector geometry and design constitute the controlled variables. Temperature, humidity and air flow are the ambient parameters that influence the process.

The versatile nature of the process has enabled a range of different materials, starting from synthetic polymers (e.g. PCL, PLLA and PEOT/PBT, among others), natural polymers (e.g. collagen, gelatine, hyaluronate and silk, among others) and composite materials to be electrospun. These have found applications in the engineering of various tissues, such as skin (Laurencin *et al*., 2008), cartilage (Li *et al*., 2005b), bone (Yoshimoto *et al*., 2003), blood vessels (Buttafoco *et al*., 2005) and nerves (Yang *et al*., 2005a), as well as in drug delivery (Ranganath and Wang, 2008). The host of parameters that control the process also enables the modification of fibre texture (Casper *et al*., 2004; De Vrieze *et al*., 2009; Deitzel *et al*., 2001; Moroni *et al*., 2006a) (smooth, rough or porous) and orientation (random or aligned) (Aviss *et al*., 2010; Teo and Ramakrishna, 2005).

Electrospun fibres have been used as scaffolds for bone regeneration in different ways. One of the first electrospun meshes for this application was fabricated with PCL (Yoshimoto *et al*., 2003). Later studies used different polymers, such as PLA (Badami *et al*., 2006), polyhydroxybutyrate (PHB) or a polymeric blend such as (poly-3-hydroxy butyrate)-co-valerate (PHBV) (Sombatmankhong *et al*., 2007). In order to improve cell attachment and enhance the biological capability of the scaffolds, natural polymers such as collagen (Shih *et al*., 2006), chitosan (Shin *et al*., 2005) and silk fibroin (Meechaisue *et al*., 2007) were either used as stand-alone scaffolds or combined with polymers such as PCL (Ekaputra *et al*., 2009; Zhang *et al*., 2005) and PLLA

(Kim *et al.*, 2008). Additionally, fibres incorporating biological factors have been used, either by direct incorporation in the polymer solution or through co-axial electrospinning (Li *et al.*, 2006; Su *et al.*, 2012). The resulting scaffolds showed potential to enhance the differentiation of bone marrow-derived MSCs. Even more interestingly, Kumar *et al.* (2011) showed that simply by tailoring the dimensions of electrospun polymeric scaffolds in comparison to other textile scaffolds, it is possible to achieve physical cues able to directly influence stem cell differentiation.

Bioactive inorganics have also been electrospun into fibres. Studies have reported the production of different ceramic fibres, such as HA (Dai and Shivkumar, 2007; Kim and Kim, 2006; Wu *et al.*, 2004) and fluorinated HA (Kim and Kim, 2006), using calcium nitrate and triethyl phosphite as precursor compounds and mixing it with a polymer such as polyvinyl alcohol (PVA) or polyvinyl butyral (PVB). After electrospinning, the polymer phase is removed by calcination. Bioglass fibres with sub-micrometre diameter were also obtained by mixing the glass with a polymeric binder (Kim *et al.*, 2006a). Cell viability and osteogenic differentiation after seeding rat MSCs were higher on Bioglass than on electrospun PCL fibres. Sakai *et al.* (2006) electrospun ultra-fine silicate fibres and evaluated the possibility of using them as scaffolds in bone regeneration by assessing cellular response with human osteoblastic cells and apatite formation in SBF. Going a step further, composites of different calcium phosphate ceramics (Jose *et al.*, 2010; Kim *et al.*, 2006b; Song *et al.*, 2008; Thomas *et al.*, 2007; Venugopal *et al.*, 2008; Yang *et al.*, 2009) with synthetic or natural polymers were also electrospun. For example, Yang *et al.* (2009) prepared composites of nano-HA with PCL, while Kim *et al.* (2006b) prepared composites of HA with PLA using a surfactant. In both studies, composite fibres showed increased osteogenic differentiation of cells compared to polymeric scaffolds alone, indicating the benefits of adding HA to the scaffold. Other studies involved electrospinning of collagen and HA composites (Song *et al.*, 2008; Thomas *et al.*, 2007), thereby mimicking the chemical composition of bone. Aligned multicomponent fibres of PLGA–collagen and nano-HA were fabricated by varying the percentage of nano-HA (Jose *et al.*, 2010). The addition of nano-HA improved mechanical properties at lower concentrations, but proved detrimental at amounts $> 0.5\%$. Preliminary cell data also showed good attachment of human MSCs on the multicomponent fibres. The challenge in such an approach is to find a compromise between the processability of the material and the level of bioactivity that it possesses.

A strategy used for improving the bioactivity of electrospun scaffolds for different regenerative applications while retaining their synthetic character is surface modification. This is, for example, achieved by plasma or by the attachment of functional groups (Chen and Su, 2011; Martins *et al.*, 2009; Park *et al.*, 2007; Prabhakaran *et al.*, 2008; Xu *et al.*, 2011) in order to control biological phenomena such as cell adhesion and differentiation as a consequence of changes in surface topography, wettability and chemistry. Treatment methods such as plasma are simple and inexpensive and can be performed in most laboratories without the need for complex equipment. The use of inert and reactive gases, such as argon and oxygen, respectively, can induce an increase in surface topography and changes in surface chemistry (e.g. increase in hydroxyl and carboxylic groups). This was shown to be responsible for enhanced osteoblast adhesion, proliferation and differentiation (Martins *et al.*, 2009). We have also investigated the use of oxygen gas plasma to tailor the surface roughness of PEOT/PBT electrospun scaffolds and showed that a roughness of 10–30 nm was associated with a significantly increased adsorption of proteins from the culture media and osteogenic differentiation of seeded human MSCs (Nandakumar *et al.*, 2013b). As an alternative to plasma treatment, we recently employed nano-imprinting lithography to provide electropun fibres with geometrically closely defined microtopographies, and demonstrated enhancement in cell–material interactions in terms of cytoskeleton organization. Osteogenic differentiation of hMSCs was also enhanced upon culture on microgrooved electrospun fibres as compared to fibres without such grooves (Nandakumar *et al.*, 2013c). Although recent studies have reported the potential of controlling cell fate by controlling surface properties in terms of level of order of topographical features (McMurray *et al.*, 2011) and their shape (Unadkat *et al.*, 2011), these technologies have been applied to 2D films, and their translation to 3D is far from trivial. Modifying surface topography features of electrspun meshes by plasma or other techniques, as described above, is a first step towards rendering 3D constructs instructive in a controlled manner.

Electrospinning offers advantages such as ease of use, fabrication of fibres in the nanometre–micrometre range with different surfaces and alignment, mimicking of the fibrillar nature of the ECM and spinnability of various materials. Although these studies inevitably depict the potential of electrospinning as a scaffold fabrication technology to produce instructive scaffolds for bone regeneration, electrospun scaffolds generally lack mechanical properties required for this application. While a few recent studies (Deng *et al.*, 2011; Wright *et al.*, 2010) have fabricated 3D electrospun scaffolds by rolling sheets into cylindrical structures to achieve structures with compressive moduli in the range of trabecular bone (lower range 20 MPa; Athanasiou *et al.*, 2000), the use of electrospun scaffolds as stand-alone supports in compressive load-bearing applications is a major limitation, as insufficient mechanical support might lead to excessive deformation, ultimately resulting in failure of nascent tissue formation (Hollister, 2005). It is possible that electrospun scaffolds will find a use in non-load-bearing bone sites, whereas they would have to be combined with more mechanically rigid structures for weight-bearing applications.

6. Injectables, putties and mouldable constructs

As mentioned earlier, another requirement from the clinic that should be taken into consideration when developing

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synthetic 3D alternatives to natural tissue is the possibility of applying the graft through minimally invasive surgery, by facilitating handling, delivery, containment and resistance to bleeding and irrigation in the defect. This would allow the treatment of both small and large, critical-sized defects in a more efficient manner, with less discomfort and infection risks for the patients and reduced healthcare costs. Although the scaffold fabrication technologies discussed so far offer the possibility of customizing the shape, bioactivity and mechanical properties of the resulting 3D scaffolds for bone regeneration, the challenge remains to implant them through minimally invasive procedures without compromising bioactivity and mechanical properties. While organic and inorganic cements have a long history of use in orthopaedic and maxillofacial applications, as discussed in various reviews (Ambard and Mueninghoff, 2006; Bohner, 2001; Chow, 2009), here we focus on more novel methods to create non-setting substitutes that preserve their 3D configuration in the defects, i.e. interconnectivity and grain or granule size optimal for cell and tissue growth. For this purpose, injectable or mouldable systems, i.e. physical blends of osteoconductive/osteoinductive particulates suspended in an 'inert' gel carrier ensuring the desired rheological properties, could offer interesting possibilities. In a first instance, extensive efforts have been put into developing mouldable or injectable formulations of DBM that basically consist of autologous or allogeneic bone grafts that have been deprived of their mineral components, thereby leaving the natural collagenous ECM, including entrapped growth factors such as BMP-2 and BMP-7. In a classical approach, calcium sulphate putties were used to deliver DBM alone or in combination with bone chips in a canine non-load bearing orthotopic defect. The injectable system was shown to restore bone defects after 6 weeks (Turner *et al*., 2003). Sodium carboxymethylcellulose and poly(ethyleneimine) were used as carriers for DBM and were shown to maintain the hydrogel shape over 1 month *in vitro* and *in vivo* (Kim *et al*., 2009); however, to what extent the bioactivity of DBM is maintained in such a system needs to be further investigated. Despite these initial promising scientific results and the development of several commercial injectable formulations, the combination of DBM with gels does not solve one of the main drawbacks of DBM, which, being a natural polymer, is still related to donor-to-donor variability, availability and disease transmission (Dinopoulos and Giannoudis, 2006). To overcome these drawbacks, synthetic inorganics, including calcium phosphate ceramics and bioglass, have been used as DBM alternatives in injectables and putties. Several inorganic formulations are clinically available in orthopaedics and maxillofacial surgery, the carrier compositions of which are closely comparable to those of DBM carriers from both natural and synthetic origins (e.g. carboxymethylcellulose, collagen, glycerin, polyethylene glycol, alkylene oxides, fibrin glue, sodium hyaluronate, collagen) (Bohner, 2010). Furthermore, putties consisting of carboxymethylcellulose, collagen and, in some cases, calcium phosphate granules have been considered as carriers of BMP-7 for clinical use (Cook *et al*., 2005; Grauer *et al*., 2001).

In addition to providing the required handling properties, the carrier should be biocompatible, resorbable and not hindering bioactivity in terms of osteoconductivity and osteoinductivity of the ceramic or bioglass particles. Barbieri *et al*. (2011) compared *in vivo* and *in vitro* putties using identical calcium phosphate particles with different carrier compositions, and established a direct relationship between carrier resorption kinetics and retention of the initial osteoinductive potential of the calcium phosphate particles: the faster the resorption of the carrier, the better the bone-forming ability of the ceramic particles is retained. Apart from the rate of degradation, carrier chemistry and the related resorption mechanism (e.g. cellular degradation, dissolution) have also an important role. Davison *et al*. (2012) compared *in vivo* two carriers with identical resorption kinetics, both of which consisted of one polysaccharide, glycerol and identical TCP granules. Despite close similarities, the authors showed that the carboxymethylcellulose–glycerol–TCP carrier allowed bone formation similar to that of TCP granules alone, in contrast to the xanthan–glycerol–TCP formulation, where only a very limited amount of bone was formed. Despite the rapid resorption of the carboxymethylcellulose–glycerol–TCP carrier, it was observed that TCP granules were well retained in a transcortical bone defect after 12 weeks of implantation in a canine model, while no traces of xanthan–glycerol–TCP putty were found after 12 weeks of implantation in identical defects. Therefore the chemistry of the carrier and associated resorption kinetics and mechanism have a critical role for retaining the bioactivity of the particulates.

In some studies, it was shown that the preclinical performance of the carrier/ceramic blends was better than the performance of ceramic granules alone, suggesting a positive effect of the carrier on bioactivity (Kania *et al*., 1998). In other studies, carrier components were shown to have a positive, although ancillary, role in bioactivity of the putty formulations. For example, Cook *et al*. (2005) highlighted a positive effect of addition of carboxymethylcellulose into a BMP-7 putty for supporting bone formation in 2.5-cm osteoperiosteal critical size ulna segmental defects (Cook *et al*., 2005), while the implantation of the same carboxymethylcellulose carrier alone into a rabbit posterolateral spinal fusion model did not lead to bone formation (Grauer *et al*., 2001). Hypotheses attempting to explain the positive ancillary contribution of the carriers to the bioactivity of injectable/mouldable formulations are numerous, as is the number of the existing carriers. Most of these can be linked to physical aspects, such as shape retention, defect containment, guidance of cells through the graft or hydrophilicity, although sometimes direct biological effects are considered, such as the presence of biologically active molecules, as in fibrin carrier.

Because putty and injectable configurations are multicomponent, the industrial development is challenging in terms of finding sterilization method(s) compatible to all components, packaging and tools to deliver the graft to the defect in a 'ready-to-use' fashion and the stability of the putty in time (sterility, handling, osteoinduction/osteoconduction potential). While DBM, bioactive glasses,

calcium phosphates and growth factors are prone to degradation in a humid environment for a prolonged time, most carriers are water-based gels (Bohner, 2010; Kanayama *et al*., 2006). Using such water-based gels in 'ready-to-use' configurations can be detrimental to the initial specifications of the osteoinductive/osteoconductive biomaterial (Davison *et al*., 2012; Schmitt *et al*., 2002) and subsequently to performance in bone regeneration. As a consequence, several clinical formulations are provided in separate vials to be mixed in the operating suite prior to application in the patient (Bohner, 2010; Kanayama *et al*., 2006), increasing the risks of infection or inadequate preparation or dosage. To overcome this bottleneck, one of the novel advances is formulations based on water-free putties, ensuring the retention of bioactivity of the bone graft substitute (Davison *et al*., 2012). This initial work on water-free carriers combined with calcium phosphate granules can be translated to other osteoinductive/osteoconductive biomaterials or molecules, such as DBM, BMPs or bioglasses, for a truly 'plug-and-play' bone graft system.

Despite these advances in the engineering of injectable and mouldable synthetic formulations, these systems still suffer from poor mechanical properties that are far from matching those of trabecular or cortical bone, making this an intrinsic drawback so far in all such injectable systems for minimally invasive surgeries for bone repair.

7. Combining technologies to create improved scaffolds

As described in previous sections, different techniques exist to build 3D constructs from a variety of biomaterials, each with its advantages and disadvantages. Just like different materials can be combined into one construct, to exploit intrinsic positive properties of each of them, combining different scaffolding technologies to build these constructs is an interesting approach. For example, a combination of RP and ESP techniques could result in a scaffold that is mechanically suitable for use in orthopaedic applications and still contains micro-/nano-scale functionality, due to the presence of ECM-like fibres. Vaquette *et al*. (2012) used this approach to create a scaffold for the regeneration of alveolar bone and periodontal ligament, where the RP scaffold was used as a bone compartment and the ESP mesh for the regeneration of the ligament. The resulting scaffold supported bone formation as well as ligament tissue formation. Furthermore, the integration of the two scaffolding technologies was also correlated with the improved attachment of the scaffold to the dentine layer apposed to the construct in a subcutaneous rat implantation model. Similarly, we combined different RP technologies to create osteochondral scaffolds with mechanical, structural and physicochemical properties matching those of articular cartilage and subchondral bone. The resulting scaffolds showed to support osteochondral tissue formation when seeded with MSCs and implanted subcutaneously in nude mice for 1 month (Moroni *et al*., 2008).

Conventional ways of producing ceramic–polymer composites include the use of blends of the two as a starting material for scaffold fabrication. In our recent study, we have compared such monolithic scaffolds produced by 3DF deposition of a polymer–ceramic blend, to scaffolds with comparable architecture with distinct polymeric and ceramic phase. The latter scaffold was prepared by building polymeric scaffolds using 3DF deposition, into the pores of which particles of the ceramic were press-fit inserted (Figure 3). The differences between these monolithic and assembled constructs lay in both the mechanical properties and the availability of calcium phosphate to the environment, which has consequences for biological

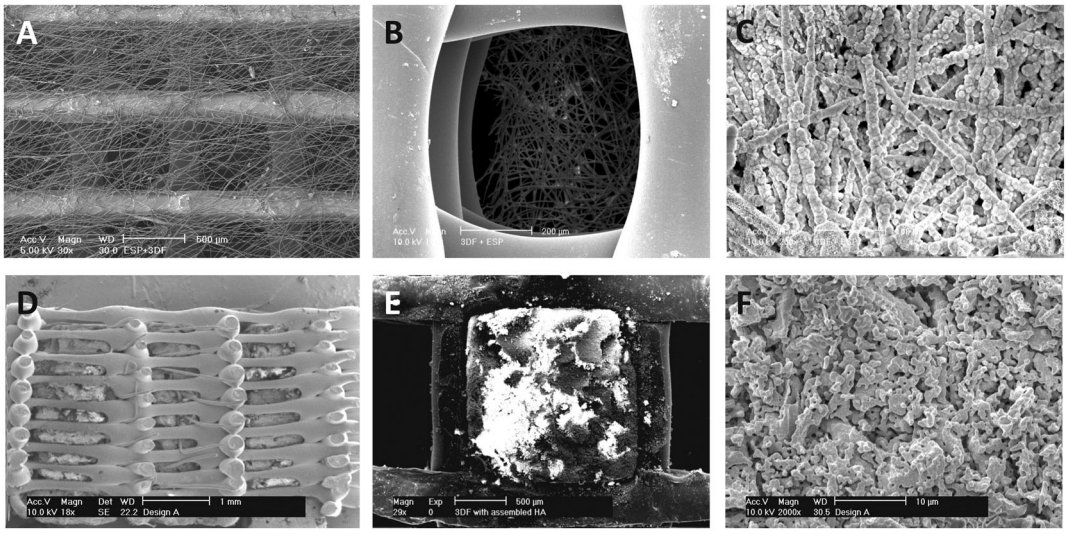

Figure 3. Scanning electron microscopy shows different examples of scaffold fabrication technology integration. Electrospinning and additive manufacturing can be combined to create scaffolds with macro-, micro- and nanoscaled fibre dimensions (A, B). These fibres can be further functionalized by calcium phosphate-based coatings (C). Alternatively, ceramic particles of customized dimensions (D, E) and microstructure (F) can be designed to fit within the pores of an RP scaffold, resulting in an alternative strategy to create composite scaffolds

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performance, while the ratio of the two material types remained the same in both approaches (Nandakumar *et al*., 2013a). Furthermore, by varying the geometry of the ceramic particles introduced in the polymeric matrix and the amount and position of these particles in the pores of the matrix, the stress and strain at break, as well as the compressive and bending modulus, could be tailored (Moroni *et al*., 2008). A similar approach was earlier used by Li *et al*. (2007) to build constructs consisting of a ceramic core, made by a conventional porogen-based technique, and a titanium alloy shell produced by RP in an attempt to retain the mechanical properties of the metal as well as the bioactivity of the ceramic. This study indeed showed more bone formation in constructs containing the ceramic core as compared to metallic scaffolds. With the aim of regenerating complex tissues affected by periodontal disease, Carlo Reis *et al*. (2011) developed a semi-rigid bilayered PLGA–CaP construct, consisting of a porous inner structure and a continuous outer membrane. The porous core was produced by combining a classical solvent-based method for preparing a polymer–ceramic composite, followed by porogen leaching and coating with a thin layer of the second CaP phase, using immersion in an aqueous solution, while the continuous membrane was produced by casting of the composite solution. The authors observed new cementum, bone and periodontal ligament formation with Sharpey fibre insertions upon implantation of these bilayered constructs in class II furcation defects in dogs, in contrast to a control group (Carlo Reis *et al*., 2011). Similar porous composite constructs, without the continuous membrane, were used for either augmentation or preservation of alveolar bone height upon tooth extraction in a clinical trial (Davies *et al*., 2010).

Considering that bioactivity of calcium phosphate ceramics is suggested to lie in dynamic processes of dissolution/reprecipitation occurring on the surface, availability of the surface to the biological environment is of importance. Coating 3D structures of different materials with a thin layer of calcium phosphate is a relatively simple approach to add bioactivity to materials such as metals and coatings. This strategy has been widely exploited to improve osteointegration between metallic hip implants and the surrounding bone (Havelin *et al*., 2000). While plasma spraying is generally accepted as a successful method for coating metallic implants, this line-of-sight process, which takes place at very high temperatures, is not suitable for coating polymers or for depositing thermally unstable calcium phosphate phases, and it cannot be applied to geometrically complex porous 3D shapes. Several other methods have also been used for coating substrates with calcium phosphate. These include sol–gel coatings (Kim *et al*., 2004), pulsed laser deposition (Cleries *et al*., 2000), radio-frequency sputtering (Yang *et al*., 2005b) and electrochemical methods (Kumar *et al*., 1999). In the 1990s, Kokubo and co-workers developed mineralizing solutions based on physiological fluids (Abe *et al*., 1990; Kokubo *et al*., 1990) that could be used to deposit apatitic layers on various substrates at near-physiological temperature and pH. This biomimetic coating method offers several advantages, such as coating of temperature-sensitive substrates such as polymers (Du *et al*., 2002b), formation of phases other than HA, such as carbonated apatite and octacalcium phosphate (OCP) (Leeuwenburgh *et al*., 2001), and deposition on porous and complex geometric shapes. Barrere *et al*. (2002a, 2002b) modified the initial biomimetic coating process by using a supersaturated SBF to increase the speed of the coating process to coat titanium-based substrates. Recently, such a coating process has also been used to coat other substrates, such as spider silk (Yang *et al*., 2010), and synthetic polymeric 3D scaffolds made using conventional (Carlo Reis *et al*., 2011; Davies *et al*., 2010; Du *et al*., 2002a), RP (Oliveira *et al*., 2009; Yuan *et al*., 2001), ESP (Yang *et al*., 2008) and combined RP + ESP techniques (Figure 3).

8. Conclusion and future perspectives

In order to exploit the power of various available technologies to a greater extent, combining or integrating them when fabricating a single scaffold clearly offers new opportunities. Advances in additive manufacturing technologies will likely deliver to the scientific and clinical communities 3D constructs with an increasing degree of complexity. In our view, it is not too optimistic to envision the fabrication of a 3D structure displaying a perfect copy of osteons arrangement and, in parallel, controlling in space and time mechanical, physical, chemical and biological cues with an exquisite precision to mimic a targeted bone segment to regenerate. A key question to answer in the immediate future is to what extent we need to achieve this *in vitro* or *in vivo*. Even from a clinical translation perspective, a relevant question that will need to be answered is to what extent we need to follow biomimicry approaches at all, if this complicates the path from the bench to the bedside without an appreciable and sensible improvement in the regeneration of critical-sized bone defects. Nevertheless, biomimicry approaches will surely be useful to obtain 3D *in vitro* models to study bone-related pathologies and screen new medical treatments. Such screens, in turn, have the potential to increase the speed at which the synthetic bone graft substitutes are developed. Therefore, the efforts of engineers to advance the existing technologies and their combinations in order to provide biologists and clinicians with suitable plug-and-play systems is justified, as this will, directly or indirectly, contribute to the improvement of the life quality of patients with musculoskeletal conditions.

Another challenge, for which we expect to see tremendous efforts in the near future, is to design and engineer materials able to control biological processes such as cell fate and tissue formation without losing their synthetic character. In particular, development of methods applicable to 3D, functional synthetic implants, that allow temporal and/or spatial control over *in vivo* processes related to bone regeneration and remodelling, will be challenging but promise an enormous gain for the field of regenerative medicine.

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