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# Hard tissue regeneration using bone substitutes: an update on innovations in materials

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Bone is a unique organ composed of mineralized hard tissue, unlike any other body part. The unique manner in which bone can constantly undergo self-re-modeling has created interesting clinical approaches to the healing of damaged bone. Healing of large bone defects is achieved using implant materials that gradually integrate with the body after healing is completed. Such strategies require a multidisciplinary approach by material scientists, biological scientists, and clinicians. Development of materials for bone healing and exploration of the interactions thereof with the body are active research areas. In this review, we explore ongoing developments in the creation of materials for regenerating hard tissues.

**Keywords:** Bone substitutes; Bone tissue engineering; Bioceramics; Hydrogel; Biopolymers

## INTRODUCTION

Repair of bone defects using implanted material commenced millennia ago; ancient Peruvian and Egyptian societies used implants to heal bone defects [1-4]. The modern era of bone substitutes commenced with the attempt of the Dutch surgeon Job van Meekeren to repair a soldier's broken skull using a skull fragment from a dog [5]. Fred Albee first described autologous bone grafting, using part of the tibia to achieve spinal fusion. The Swedish surgeon Levander showed that osteoinduction could be used to induce regeneration of hard tissue [6]. Urist [7] first reported, in 1965, that bone morphogenetic proteins (BMPs) exhibited osteoinductive potential. Hard tissue repair, and regeneration science and technology, have advanced rapidly in the modern era. An in-depth understanding of the underlying principles has been attained, new methods and materials developed, and a multidisciplinary approach

used to achieve successful hard tissue regeneration. Many scaffold systems have been proposed for bone tissue engineering. Innovation have been made in all of scaffold design, material selection, incorporation of drugs and growth factors, mechanical stability, and bone regeneration efficiency. However, autografts are still considered to be the best bone graft option for hard tissue repair; synthetic bone graft substitutes do not exhibit equivalent osteogenic or osteoinductive performance. However, autografting does not meet the overall medical demand for orthopedic implants. Harvesting of adequate quantities of bone is difficult and postoperative complications occur at harvest sites. Allografts and xenografts are both good alternatives, but are associated with risks of disease transmission and immunorejection. Thus, synthetic bone graft substitutes are the logical option when it is sought to meet the rapidly increasing demand for orthopedic implants, even though synthetic bone substitutes have some in-

herent limitations in terms of strength, osteoconduction, osteoinduction, osseointegration, and biodegradation. Current studies on bone substitutes are focused on improving various features of scaffolds; and include the development of new biomaterials, modification of mechanical and structural-morphological features, enhancement of biocompatibility by chemically modifying the surfaces of materials, improvement of osteoinductive capabilities and the ability to incorporate growth factors, and loading of stem cells onto scaffolds to induce self-initiated tissue regeneration. These remarkable advances have helped reduce the gap between autografts and synthetic bone graft substitutes.

Key issues in successful implantation are the initial and long-term immune reactions of the body to the implant. The immune system recognizes the implant, and may reject it, initiating many physiological responses involving immune cells. Thus, the chemical nature of the implant material is key to its biocompatibility. Consequently, cell-material interactions within the defect zone determine the overall success of healing. Hard tissue repair also requires high-level mechanical stability; this is not the case for other injured tissue. Load-bearing capacity and structural rigidity is afforded by the skeletal system, and repaired hard tissue is directly subjected to or is expected to tolerate significant mechanical loading, which limits the choice of bone substitute materials. Thus, only a few materials are presently considered useful. Hard tissue is composed of carbonated hydroxyapatite (HAp) crystals and collagen (the principal building blocks) with cellular and systemic components. Thus, calcium phosphate ceramics and collagen are natural choices of bone substitutes. Positive cell-material interactions are also observed with several other inorganic materials like bioglasses, phosphates of magnesium; sulfate, carbonate, and silicate of calcium. Some very inert inorganic materials, including alumina, zirconia, titanium alloy, and cobalt-chromium alloy, find specific hard tissue applications, but these materials are nonresorbable and osseointegration is absent at the bone-implant interface. Synthetic biodegradable polymers, including polylactic-co-glycolic acid (PLGA), polycaprolactone (PCL), and polyethylene glycol (PEG), interact positively with cells, and are used as substitute bone scaffolds [8,9]. These materials are degradable in the physiological

environment and the degradation products have no harmful effects. Moreover, degradation rate, hydrophilicity, and mechanical strength can be controlled by manipulating the chemical composition. Many natural biopolymers are also available, and are very suitable bone substitutes in terms of cell-material interactions. Chitosan, alginate, cellulose, gelatin, collagen, keratin, and hyaluronic acid are inherently recognizable by cells and exhibit favorable cell-material interactions. These are large polymers of very high molecular weight. Biodegradation of such molecules is very rapid, and the degradation products may stimulate the physiological mechanisms of healing. Bone substitute materials are currently selected based on an ability to impart additional biocompatibility to a structurally stable scaffold [10,11].

Healing of bone defects in adults closely resembles bone formation during organogenesis. Most fractures heal by indirect or secondary fracture healing, via formation of an intermediate callus [12]. An inflammatory response occurs soon after a fracture of bone, or surgical intervention, and extravascular blood cells form a blood clot. After this initial immune reaction, collagen fibers and mineralized osteoids combine to form a soft callus around the injury site. This soft (or fracture) callus ossifies to form a disorganized structure termed woven bone. During a later phase of bone formation, this woven bone is replaced gradually with highly organized lamellar bone, which begins to form soon after the collagen matrix of either tissue becomes mineralized. Osteoblastic cells penetrate the mineralized matrix and angiogenesis begins with creation of microvessels. Osteoblasts deposit new lamellar bone on the surface of the mineralized matrix. Eventually, all woven bone and the fracture callus are replaced by lamellar or trabecular bone. This remodeling process transforms trabecular bone into natural compact bone Said so, the whole process should occur inside a bone substitute scaffold without jeopardizing the series of events and occasionally complementing the process by its morphological and chemical attributes.

Bone substitute scaffolds must meet stringent requirements; they must be nontoxic, mechanically sound, have a three-dimensional (3D) porous structure, exhibit optimum biodegradation, allow new bone formation at an acceptable rate, be economical to make,

and allow easy fabrication into the final preforms [13-15]. Scaffold architecture is critical to optimize the microenvironment for the synthesis of new tissue, and to allow flow or diffusion of nutrients between cells and the surrounding environment. Scaffold properties depend primarily on the biomaterial used and the fabrication process. Several ceramic and glass materials have superior biocompatibility but poor mechanical strength and stability, rendering them unsuitable as porous scaffolds for bone tissue regeneration. Apart from their lower intrinsic strength, processing defects (such as irregularly shaped pores), surface defects, and residual stress, all lower the mechanical strength of the scaffold systems made of these materials. Thus significant research is devoted to come up with stronger and more biocompatible systems.

Recent advances in bone substitutes have made significant progress regarding these challenges. Advances in materials design; chemical modification; fabrication techniques creating stronger, more porous, and more biocompatible scaffolds; combinations of various strategies to enhance cell-material interactions; and stimulation of cells to ensure rapid but controlled bone regeneration, are continuously reported. Tissue engineering has opened a new dimension in bone substitute technology.

The aim of this review is to explore modern frontiers of bone substitute technologies. We will explore how the technology is shaping its current form. Our discussion broadly covers innovations in materials development and fine-tuning, together with structural and functional improvisations.

## INNOVATIONS IN MATERIALS

As discussed earlier, the chemical nature of the scaffold material is fundamental for successful implantation. Cell-material interactions govern the adaptation and systemic integration of the foreign body into the physiological environment. Many choices of material are available; each has advantages and disadvantages. Thus, ever-higher performance bone substitute systems (in terms of both mechanical and biological properties) are under continuous development. Some innovations are discussed below under different categories of material.

### Bioceramics

Bioceramics are the best-studied bone substitute materials because they are chemically similar to bone. Fabrication of bioceramic porous scaffolds is achieved using various techniques to create pores, including salt leaching [16], sponge replica and gas foaming [17-20], porogen-based method [21], 3D printing [22], etc. Fig. 1 shows a scaffold fabricated by sponge replica method.

Both the microstructure and pore size and porosity significantly influence the mechanical properties and osseointegration of a scaffold [23]. Newer fabrication techniques have been proposed which allow greater control of pore size, porosity, scaffold shape, ease of fabrication, and reliability of physicomechanical properties. Recent fabrication techniques include 3D printing [24], stereolithography [25], *in situ* synthesis using a reactive phase [26], and laser cladding [27]. These methods are particularly useful for creating customized scaffolds with predictable mechanical performance. Moreover, these methods afford new opportunities for development and customization of scaffolds, allowing achieve greater control over cell-material interactions in the biological environment.

The bioactive concept seeks to balance mechanical strength and bioresorbability using biphasic calcium phosphate (combination of hydroxyapatite and tricalcium phosphate). This material is under intense study in terms of chemical modifications, for use as a base material allowing further functionalization via surface treatment, as a component of hybrids, and in terms of loading of bioactive secondary phases. Calcium phosphates have been chemically modified
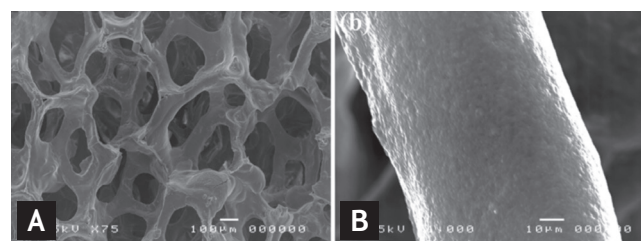


**Figure 1.** Scanning electron micrographs of biphasic calcium phosphate scaffolds fabricated using the sponge replica method. Low magnification image (A) and high magnification image of the scaffold strut (B).

by incorporating Si, Sr, and Zn [28-35]. These chemical modifications enhanced osteoblastic proliferation and material performance; dissolution rate, densification behavior, mechanical strength, and biocompatibility. The ions promote bone formation whern the ions are present in dissolution products adjacent to the cell-material interface.

Nanophase bioceramics have unique advantages; size effects and nanoscale surface phenomena are in play. These materials may be used as fillers in polymeric scaffold systems, to improving both mechanical and biological properties, and to coat on metallic implants [36]. Various synthetic processes are used to prepare nanophase calcium phosphate, modify process chemistry and powder characteristics (in terms of morphology and biocompatibility). Hydrothermal, sol-gel, wet chemical, and biomimetic deposition methods have been investigated [37].

A key issue with bioceramic bone substitutes is the fact that load-bearing is limited during healing. However, ceramics are historically regarded as nonload-bearing materials. Bioceramics, such as HAp, tricalcium phosphate (TCP), and other calcium phosphate materials, including calcium sulfate, are brittle. Introduction of pores significantly decreases bulk strength. However, high-level porosity is indispensable for sound osteointegration, and efforts are being made to modify the microstructures and structural features of porous bioceramics, to achieve higher strength. Processing conditions and methods drastically affect the surface characteristics of fabricated bioceramics scaffolds; paving the way for construction of high-strength scaffolds. Bone substitutes of block, cylindrical granule, and spherical granule types have been developed using sponge replica, fibrous monolithic, and slurry drip processes [38-41] Fig. 2 shows a multichannel granular bone substitute and its internal microstructure. Bone formation with angeogenesis using this type bone substitutes are described in the schematic model.

The use of ceramic-polymer hybrid systems to fabricate scaffolds has attracted much attention. However, the choice of polymeric materials is increasing only gradually. The use of polymers alone may not be optimal when it is sought to create biocompatible bone substitutes with adequate mechanical strength.
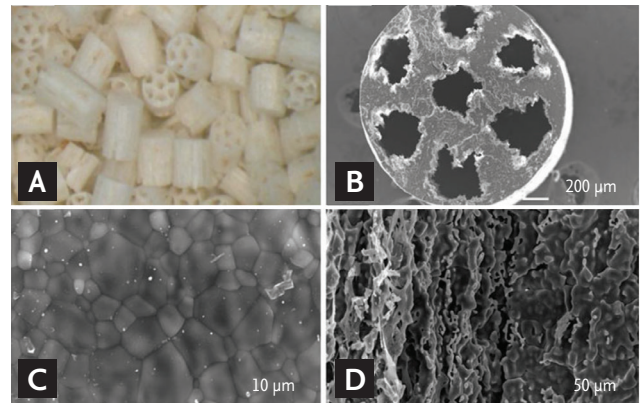


**Figure 2.** Optical microscopic (A) and SEM images (B) of granular bone substitutes. Frame (C) and pore (D) surface of the granular bone substitute. Adapted from Byun et al., with permisson from Springer [39].

Calcium phosphates, and (in some cases) bioglass and glass ceramics, combined with polymers, afford good mechanical properties and high-level biocompatibility. Bioceramics may be added to reinforce the matrix, improve both mechanical characteristics and biocompatibility of synthetic biopolymers that do not exhibit adequate levels of cell- material interaction [42-44].

Ceramic-polymer hybrid composite systems enhance the morphological and functional properties of scaffolds. Usually, ceramic-only scaffolds are prepared via high temperature sintering to ensure strength and stability. This prohibits *in situ* functionalization by biochemical agents, such as drugs and/or growth factors, and hampers replication of any biomimetic process, such as co-deposition and co-precipitation, that occurs in the physiological environment during natural bone regeneration. HAp nanocrystals serve as the chief building blocks of natural bone. Thus, thermally prepared ceramic scaffolds are entirely different from those prepared in a low-temperature environment. Biomimetic scaffold fabrication has been investigated in the context of ceramic-polymer hybrid systems [45,46]. Incorporation of active biomolecules, including growth factors, drugs, and even genes, is of great interest (please see below).

### Glass and glass ceramics

Bioactive glass and glass-ceramics exhibit superb biocompatibility and can directly bond to living tissue [47,48]. Bioactive glass is amorphous, whereas glass-ce-

ramic is a crystallized glass (the crystalline phase is created during thermal treatment) with a residual glass phase. Both materials trigger specific biological responses that enhance cell-material interactions. The products of rapidly degrading bioglass materials up-regulate gene expression to directly promote cellular activity, accelerating bone regeneration and formation of natural bonds with existing bone. The bioactive and bone-bonding mechanisms of 45S5 glass (developed by Professor Hench) have been widely studied and are described in detail elsewhere [49,50]. The best bioglass occupies a narrow range in the ternary phase diagram of $Na_2O$-CaO-$SiO_2$, with a constant $P_2O_5$ level. Several modifications (via addition of $B_2O_3$, $TiO_2$, $Li_2O$, FeO, and/or SrO) have been proposed [51-57]. All materials are prepared by melt quenching; the molten phase is quenched to stabilize the glass structure at room temperature. Fabrication of a bioglass scaffold bone substitute requires thermal reprocessing, triggering crystallinity and disruption of the glass structure. The glass phase is the key to biocompatibility; disrupting the phase has adverse effects. Thus bioglass scaffolds made via thermal reprocessing exhibit decreased biocompatible. Either a glassy or crystalline phase may form, depending on the nature of Si-O bonding in the glass structure. Nonbridging Si-O (compared to the bridging Si-O bond of $SiO_2$) allows bioglass to dissolve in aqueous environments; bioactivity follows. Sintering of bioglass changes nonbridging Si-O bonds to bridging Si-O bonds. Addition of $K_2O$, MgO, $B_2O_3$, and/or $Al_2O_3$ allows bridging Si-O bonds to be retained at higher sintering temperatures [58]. Several modified systems are already available, including 13-93, ICIE16, and BioK [59-61]. In a recent attempt bioactive glass system has been synthesized using conventional $SiO_2$-CaO-$Na_2O$-$P_2O_5$ composition, employing an ultrasound-assisted hydrothermal method [62].

Bioglass systems were prepared by sol-gel processing to avoid thermal treatment. This process allows creation of nanophase and nanoporous systems. This system invites entirely new applications in drug and growth factor delivery; the scaffolds are rapidly biodegraded, and thus offer enhanced biological responses [63]. Compositional variation has been reported during sol-gel processing [64-67]. Sol-gel bioglass of high silica content can be prepared in the ab-

sence of network modifier cations. Bioglass of similar composition to the melt-derived counterpart is also available. As stabilization of glass via conventional heat treatment alters glass properties, including particle size and density, stabilization of sol-gel-derived glass at room temperature would aid in biocompatibility. Such properties are also significant in the field of composites; bioactive glass powder is used to reinforce polymeric matrices of a low elastic modulus [68,69]. Textural features, including particle size distribution, specific surface area, and porosity, strongly influence bioactivity. Thus, the rate of formation of the interfacial hydroxyl carbonate apatite layer, which is structurally and chemically equivalent to the mineral phase of bone, is influenced by particle size range and the powder volume fraction during bone-bonding.
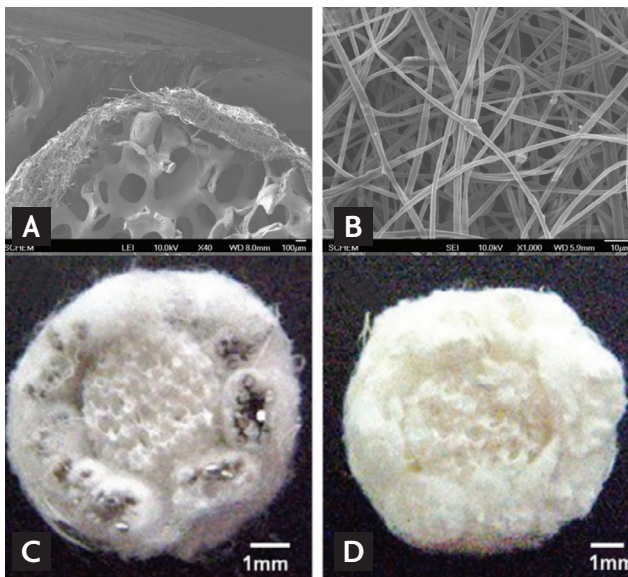
Bioglass of excellent bioactivity has been used to coat the surfaces of less biocompatible or bioinert materials, such as titanium or steel. Metallic materials are the first choice when mechanical stability is desired. However, the inherent lack of any direct bond with natural bone poses a significant postoperative risk of implant loosening and friction damage to surrounding tissue. Various methods have been used to modify metallic implant surfaces via coating with bioglass. Surface modification can also be achieved using calcium phosphate materials. Plasma spraying, electrophoretic deposition, and dip coating methods have been used to coat metallic scaffolds [70,71].

## Biopolymers and hydrogels

The most diverse range of materials for hard tissue regeneration are biopolymers. Many such polymers are naturally derived and thus, are inherently safe. Many natural polymers have been used for bone tissue engineering; these include chitosan, hyaluronic acid, alginate, oxidized alginate, gelatin, pectin, starch; and proteins including soy, collagen, fibrin gels, and silk [72]. Synthetic polymers, including polyglycolic acid, polylactic acid, their copolymer PLGA, polyanhydrides, polycarbonates, polyphosphazenes, polyfumarates, and poly(butylene terephthalate)/poly(ethylene oxide), have also been used extensively among others [73-75].

Biopolymers are degradable under physiological conditions and the degradation products are metabolically discarded. Natural biopolymers have been used to
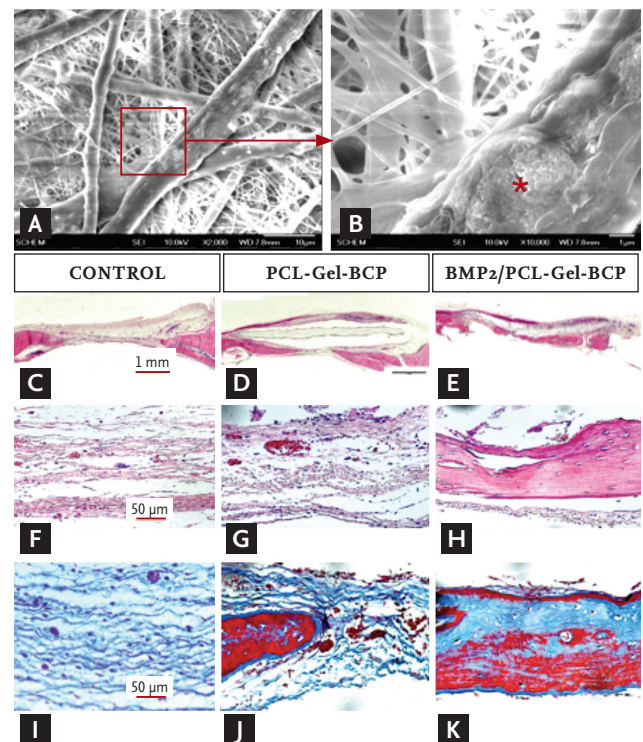
manufacture porous scaffolds. All natural polymer systems exhibit poor mechanical stability; and must thus be coupled with natural polymers and/orsynthetic biodegradable polymers to create stable systems. Composites have been used to physically or chemically crosslink and stabilize scaffolds. New fabrication techniques, (particularly those applicable at room temperature) including electrospinning and 3D printing, have rendered such approaches feasible. Various polymer scaffolds have been created via electrospinning of collagen, gelatin, PLGA, PEG, and PCL. The inherent nanoporous structure of an electrospun mat enables easy diffusion of nutrients through the scaffold. The high surface area of the mat improves tissue-material interactions. These features have been exploited to deliver growth factors and drugs enhancing bone tissue regeneration [76,77]. Modifications of basic electrospinning technology have allowed the creation of patterned mats, composite scaffolds, and complex interlayers in artificial biomimetic scaffolds [78-80]. Fig. 3 depicts a hybrid ceramic-polymer scaffold for artificial small bone with electrospun mat forming a bone conducive porous scaffold assembled around a porous zirconia core. Electrospun mats have been used to deliver drugs; the stability and biocompatibility of such mats are enhanced by the use of both synthetic and natural polymers, and HAp nanoparticles [81]. Fig. 4 showed polymer-ceramic hybrid electrospun scaffold as a career for bone morphogenetic growth factor and depicted the enhanced bone regeneration ability of the drug loaded scaffold using a rat calvarial defect model.

Natural polymers, including hyaluronic acid, collagen, gelatin, alginate, and chitosan, are obtained from animal and plants wherein they play physiologically important roles. Thus, these biopolymers are inherently favor cell-material interactions and have been widely used in bone tissue engineering [82]. These polymers undergo extensive hydration to form hydrogels under physiological conditions. Hydrogels serve as matrices



**Figure 3.** Scanning electron microscopic images of a ZrO2/biphasic calcium phosphate scaffold wrapped with poly(methylmethacrylate)-poly-ε-caprolactone-hydroxyapetite (PMMA-PCL-Hap) fibers (A), and the fibers themselves (B). (C) Bundles surrounded by PMMA/PCL/Hap; (D) the bundles after removal of the steel wires. Adapted from Kim et al. [80].



**Figure 4.** Scanning electron micrographs of polycaprolactone (PCL)-gelatin (Gel)-biphasic calcium phosphate (BCP) (A, B) electrospun scaffolds. Histological cross-sections of rat skull implanted with PCL-Gel-BCP and bone morphogenetic protein-2 (BMP-2)/PCL-Gel-BCP scaffolds, and a negative control (defect only) stained with H&E (C-H) and Masson's trichrome (I-K) 4 weeks after implantation. Adapted from Kim et al., with permission from Mary Ann Liebert, Inc. [81].

for tissue engineering, mimicking the topology of the extracellular matrix, thus improving cellular adhesion and proliferation [83]. However, hydrogels lack the mechanical strength needed for weight-bearing, which is a serious disadvantage, rendering it impossible to use them alone as bone regeneration systems *in vivo*. Hybrid scaffold systems in which ceramic scaffolds are loaded with hydrogels impart mechanical stability [84]. Hydrogels have been admixed with growth factors and drugs to enhance bone regeneration [85]. Various types of hydrogels, containing drugs or growth factors, are being actively developed, drug encapsulation and subsequent release in the required zone are goals of such work.

Bone tissue engineering will potentially overcome many drawbacks of bone substitute scaffolds, including the lack of osteoinduction, poor vascularization, and delayed healing. It is possible to create tissues (including bone) on preformed scaffolds loaded with stem cells, which can differentiate into any desired cell type. Bone marrow- or adipose tissue-derived mesenchymal stem cells (MSCs) have high proliferative capacities and can differentiate into osteoblasts; thus, they have been used in bone tissue engineering [86,87]. Tissue engineering using adipose tissue-derived MSCs is attractive, such cells are abundant and donor site morbidity is minimal. Hydrogel scaffolds are indispensable in such work. Highly swollen hydrogels can suspend cells 3D and support nutrient diffusion to the cells. Additional modifications of hydrogels seek to enhance cell homing, by improving adhesion and attachment behaviors [88-90]; thesefeatures may enhance the biomimetic environment for the encapsulated cells. Hybrid system of hydrogel loaded into β-TCP/HAp ceramic scaffold was shown to facilitate delivery and distribution of cells in a mechanically stable manner [91]. Several hydrogels, including those made of alginate, fibrin glue, hyaluronic acid, chitosan, pluronic F12, thiol-norbornene, and PEG-poly (l-alanine) thermogel, promoted bone formation induced by MSCs and osteoblasts [92-95].

Often, use of a single polymeric hydrogel system is inadequate to overcome the drawbacks of rapid degradation and lack of stability in the physiological environment. Polyelectrolyte complexes (PECs) are formed by reaction between oppositely charged poly-

mers [96]. Both cationic and anionic polymers are biocompatible and biodegradable, and form a PEC via weak bonding between the anionic and cationic groups enhancing the stability of the scaffold. PECs prepared from natural polymers, such as polysaccharides, have the additional advantages non-toxicity and bioabsorbability. Three-dimentional PEC scaffolds, fabricated via gas foaming, phase separation, electrospinning, or freeze-drying, have been used for cartilage repair and to reconstruct oral/maxillofacial defects [97,98]. Complexation can be enhanced by chemically modifying the functional groups via oxidization; this enhances interaction during polyelectrolyte complexation. Such scaffolds exhibit excellent degradation characteristics and form bone *in vitro* and *in vivo*. PEC scaffolds immobilize growth factors, allowing their controlled release to improve the functionality and performance of hydrogel scaffolds [96]. PEC microspheres, membranes, nanotubes, nanoparticles, fibers, and coarcevates have been developed, using different polysaccharides and polyamines [99-103]. These materials have been used for core encapsulation, surface adsorption, and matrix entrapment of various biomolecules and cells, including proteins, enzymes, and stem cells [104-107]. In particular, this approach has been successfully used to immobilize and deliver nanosized biomolecules, such as peptides and DNA plasmids [108,109].

Hydrogel systems have also been used as ECM-like materials in conjunction with HAp/TCP hybrid composites. Hydrogels can be modified in many ways to alter hydrophilicity/hydrophobicity and enhance biological activity. Freeze drying of hydrogel systems creates a macroporous microstructure enhancing cellular proliferation and growth. Composite scaffolds featuring stabilized macroporous hydrogels loaded into a macroporous biphasic calcium phosphate scaffold have been used to impart ECM-like attributes [110] affording superior bone regeneration and hydrogel stability Such a system is shown in Fig. 5 where Hyaluronic acid-Gelatin/BCP hybrid scaffold, where the biopolymer were loaded as hydro-gel, showed excellent bone formation potential.
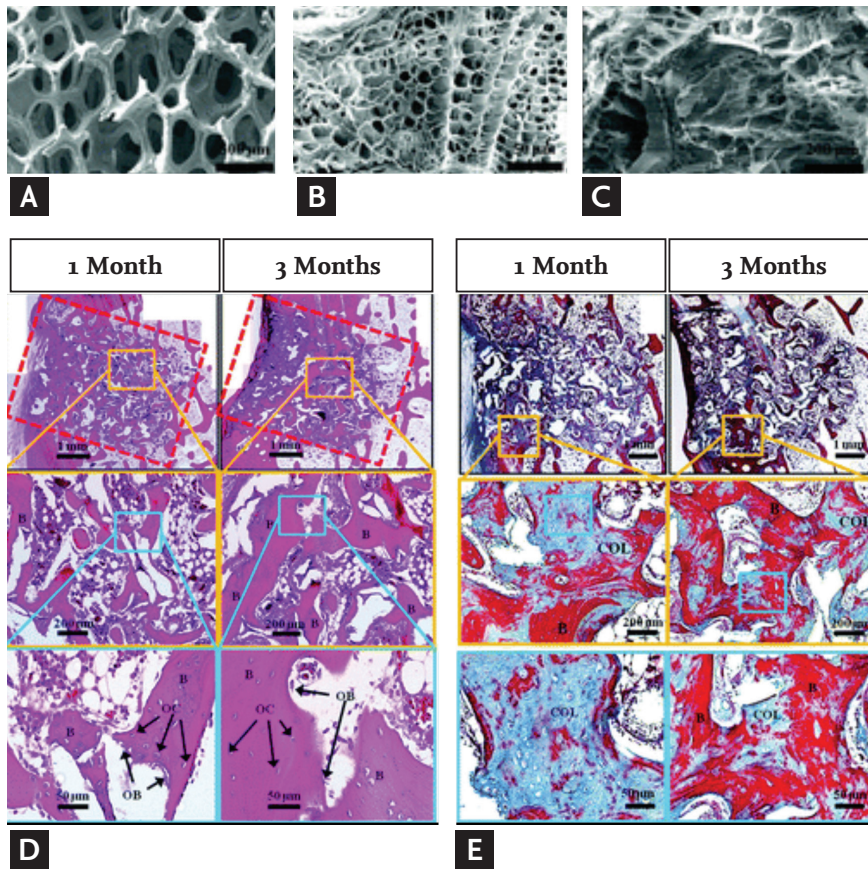
**Figure 5.** Morphologies of a sponge biphasic calcium phosphate (BCP) scaffold (A), a hyaluronic acid (HyA)-gel hydrogel (B), and a HyA-Gel/BCP scaffold (C), as revealed by scanning electron microscopy. (D) Histological sections of a rabbit femur implanted with HyA-Gel/BCP; H&E staining reveals new bone formation (B), osteocytes (OC), and osteoblasts (OB). Most pores are filled with new bone after 1 month; bone growth continued for 3 months. (E) Histological sections of a rabbit femur implanted with HyA-Gel/BCP; Masson's trichrome staining shows collagen (COL) deposited within the scaffold site (blue) and new bone formation (B) (red). Adapted from Nguyen et al., with permission from Mary Ann Liebert, Inc. [110].

## OPTIMIZING THE MICROSTRUCTURE AND MORPHOLOGY OF BIOCERAMIC-BASED BONE SUBSTITUTES

Scaffold mechanical properties, such as compressive and bending strength, sharply decrease as porosity increases. Porous TCP ceramic scaffolds also lack fracture toughness [111]. Reinforcing with particles, fibers, and whiskers can improve the mechanical properties of ceramic scaffold [112-114]. *In situ* formation of nano-HAp whisker-reinforced porous TCP scaffolds has been reported [115]. Enhanced mechanical (load-bearing) and biological performance of PLGA-coated TCP composite scaffolds has been reported [116]. Porous HAp scaffolds with functionally graded core/shell structures exhibit improved mechanical properties [117]. Calcium silicate ceramic scaffolds toughened with HAp whiskers have been used in bone tissue engineering [118]. Silk has been used as a biocohesive sacrificial binder during fabrication of HAp load-bearing scaffolds [119]. Additive manufacturing (AM) techniques have been developed to enable production of free-form porous scaffolds with custom-tailored architectures [120]. Commercially available AM techniques include selective laser sintering, stereolithography, fused deposition modeling, precision extrusion deposition, and 3D printing. Detailed descriptions of the working principles, recent trends, and current limitations of these techniques are provided in several review articles [121-123].

Porous bioactive glass scaffolds with oriented microstructures have been prepared by unidirectional freezing of organic (camphene)-based suspensions [124]. Porous material (based on spongy titanium granules) has been used in bone tissue engineering [125]. Granular bone substitutes with unidirectional channels have been fabricated using a fibrous monolithic process; the pore geometry was regular and the mechanical strength increased [126]. Granular scaffolds have been prepared by electrospraying, microemulsion, and phase-separation methods.

## CLINICALLY AVAILABLE BONE SUBSTITUTES AND CLINICAL INVESTIGATIONS

Many commercial products are available, with specific applications, such as filling of bone voids, craniofacial bone voids; and facilitation of spinal fusion. These fillers have either been submitted to the US Food and Drug Administration for premarket approval, or have such approval. HAp ceramic grafts include Cerabone (Botiss biomaterials GmbH, Zossen, Germany), Endobon (Biomet Inc., Wilrijk, Belgium), Ostim (Heraeus Kulzer, Hanau, Germany), and Pro Osteon 500 (Interpore Cross, Irvine, CA, USA). ChronOS (Synthes, West Chester, PA, USA) and Vitoss (Orthovita, Malvern, PA, USA) are both made of TCP. Composite HAp and TCP ceramic grafts include BoneSave (Stryker, Hopkinton, MA, USA) and Mastergraft (Medtronic, Minneapolis, MN, USA). Calcibon (Biomet Inc.), ChronOS Inject, HydroSet (Stryker, Hopkinton, MA, USA), and Norian SRS (Synthes) are calcium phosphate cements. Bone Plast (Biomet Inc.), MIIG X3 (Wright Medical Technology Inc., Memphis, TN, USA), OsteoSet (Wright Medical Technology Inc.), and Stimulan (Biocomposites, Staffordshire, UK) are calcium sulfate-based systems. NovaBone (NovaBone, Jacksonville, FL, USA) and Vetros (Biomedtrix, Boonton, NJ, USA) are bioactive glass-based bone substitute. Many clinical evaluations have been performed using these systems. Maxillary sinus floor augmentation, reconstruction of periodontal osseous defects and the alveolar ridge, hip replacement, and anterior cervical fusion have been performed, and the results documented. Clinical trials, clinical series, and case reports have canvassed possible applications of tissue engineering using MSCs. Clinical trials on non-unions or delayed unions treated via cell therapy have been reported [127-130]. Several studies used cells and scaffolds [131,132]. These trials and studies have established the feasibility and (reasonable) safety of cell therapy-based approaches, and provide measures of the efficacy of bone healing.

## CURRENT CHALLENGES AND FUTURE DIRECTIONS

The mechanical stability and osteointegrity of scaffolds that must bear loads long-term are critical problems. Insufficient vascularization of the interiors of thick bone substitutes, limiting cell ingrowth and survivability, is associated with poor osseointegration. Mechanical strength is heavily dependent on porosity and geometry of the scaffold, and pore and strut dimensions. These features are primary dependent on the type of fabrication process, specially for ceramics. In this case it is difficult to guarantee microstructural integrity and the required surface characteristics when the pore geometry is intricate and irregular. However, new manufacturing techniques like additive manufacturing, are promising; pore dimensions can now be precisely controlled, reducing surface irregularities on porous ceramics scaffolds. Other tissues, such as vascular and nerve tissue, must also grow to allow maturation of new bone within the porous structure. Vascular infiltration, nutrient transport, and cell migration must be optimal in any scaffold. Angiogenic growth factors and vasculogenic cell sources are being actively researched to resolve the poor vascularization of large bone graft substitutes. Bone formation involves a complex cascade of signaling pathways triggering a range of cellular and biochemical processes. Use of BMP-2 has been widely considered as highly effective to facilitate this process. However, bone tissue regeneration involves many growth factors and chemokines; the optimal mix of such materials and their synergies with other growth factors in terms of release kinetics and dosage requires further work. A controlled drug delivery system treating or preventing infection, in combination with a bone graft substitute, may allow optimal bone regeneration.

Successful clinical application of bone substitutes requires interplay among cells, biological signals, and biomaterials. Many unanswered questions and unexplored frontiers remain for the optimal use of nanostructured materials. Fundamental advances in in life and materials sciences are required.

## CONCLUSIONS

Bone substitute development is a multidisciplinary research field, and significant improvements in current options, and new developments, are likely to increase

our basic understanding of the underlying principles. New biomaterials are dramatically broadening the options for advanced therapeutic remedies. Various material systems are being modified to elicit better biological and systemic responses. However, a perfect treatment option for bone defects remains elusive; the multidisciplinary approach seeks to overcome existing problems.

## Conflict of interest

No potential conflict of interest relevant to this article was reported.

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## REFERENCES

1. Urist MR, O'Conner BT, Burwell RG. Bone Graft, Derivatives and Substitutes. Oxford: Butterworth Heinemann, 1994.

2. Flati G, Di Stanislao C. Chirurgia nella preistoria: parte I. Provincia Med Aquila 2004;2:8-11.

3. Donati D, Zolezzi C, Tomba P, Vigano A. Bone grafting: historical and conceptual review, starting with an old manuscript by Vittorio Putti. Acta Orthop 2007;78:19-25.

4. Pryor LS, Gage E, Langevin CJ, et al. Review of bone substitutes. Craniomaxillofac Trauma Reconstr 2009;2:151-160.

5. de Boer HH. The history of bone grafts. Clin Orthop Relat Res 1988;226:292-298.

6. De Long WG Jr, Einhorn TA, Koval K, et al. Bone grafts and bone graft substitutes in orthopaedic trauma surgery: a critical analysis. J Bone Joint Surg Am 2007;89:649-658.

7. Urist MR. Bone: formation by autoinduction. Science 1965;150:893-899.

8. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. Biomaterials 2000;21:2529-2543.

9. Kellomaki M, Niiranen H, Puumanen K, Ashammakhi N, Waris T, Tormala P. Bioabsorbable scaffolds for guided bone regeneration and generation. Biomaterials 2000;21:2495-2505.

10. Torres AL, Gaspar VM, Serra IR, et al. Bioactive polymeric-ceramic hybrid 3D scaffold for application in bone tissue regeneration. Mater Sci Eng C Mater Biol Appl 2013;33:4460-4469.

11. Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 2006;27:3413-3431.

12. Sfeir C, Ho L, Doll BA, Azari K, Hollinger JO. Fracture repair. In: Lieberman JR, Friedlaender GE, eds. Bone Regeneration and Repair: Biology and Clinical Applications. Totowa: Humana Press, 2005.

13. Burg KJ, Porter S, Kellam JF. Biomaterial developments for bone tissue engineering. Biomaterials 2000;21:2347-2359.

14. Goff T, Kanakaris NK, Giannoudis PV. Use of bone graft substitutes in the management of tibial plateau fractures. Injury 2013;44 Suppl 1:S86-S94.

15. Ricciardi BF, Bostrom MP. Bone graft substitutes: claims and credibility. Semin Arthroplasty 2013;24:119-123.

16. Cannilloa V, Chiellinib F, Fabbria P, Sola A. Production of Bioglass® 45S5: polycaprolactone composite scaffolds via salt-leaching. Compos Struct 2010;92:1823-1832.

17. Wu C, Zhang Y, Zhu Y, Friis T, Xiao Y. Structure-property relationships of silk-modified mesoporous bioglass scaffolds. Biomaterials 2010;31:3429-3438.

18. Wang X, Ruan JM, Chen QY. Effects of surfactants on the microstructure of porous ceramic scaffolds fabricated by foaming for bone tissue engineering. Mater Res Bull 2009;44:1275-1279.

19. Wu C, Ramaswamy Y, Zreiqat H. Porous diopside (CaMgSi(2)O(6)) scaffold: a promising bioactive material for bone tissue engineering. Acta Biomater 2010;6:2237-2245.

20. Wu ZY, Hill RG, Yue S, Nightingale D, Lee PD, Jones JR. Melt-derived bioactive glass scaffolds produced by a gel-cast foaming technique. Acta Biomater 2011;7:1807-1816.

21. Xu S, Lin K, Wang Z, et al. Reconstruction of calvarial defect of rabbits using porous calcium silicate bioactive ceramics. Biomaterials 2008;29:2588-2596.

22. Wu C, Luo Y, Cuniberti G, Xiao Y, Gelinsky M. Three-dimensional printing of hierarchical and tough mesoporous bioactive glass scaffolds with a controllable

pore architecture, excellent mechanical strength and mineralization ability. Acta Biomater 2011;7:2644-2650.

23. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials 2005;26:5474-5491.

24. Inzana JA, Olvera D, Fuller SM, et al. 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. Biomaterials 2014;35:4026-4034.

25. Brie J, Chartier T, Chaput C, et al. A new custom made bioceramic implant for the repair of large and complex craniofacial bone defects. J Craniomaxillofac Surg 2013;41:403-407.

26. Peroglio M, Gremillard L, Eglin D, Lezuo P, Alini M, Chevalier J. Evaluation of a new press-fit in situ setting composite porous scaffold for cancellous bone repair: towards a "surgeon-friendly" bone filler? Acta Biomater 2010;6:3808-3812.

27. Comesana R, Lusquinos F, del Val J, et al. Calcium phosphate grafts produced by rapid prototyping based on laser cladding. J Eur Ceram Soc 2011;31:29-41.

28. Hayakawa S, Kanaya T, Tsuru K, et al. Heterogeneous structure and in vitro degradation behavior of wet-chemically derived nanocrystalline silicon-containing hydroxyapatite particles. Acta Biomater 2013;9:4856-4867.

29. Baeza A, Izquierdo-Barba I, Vallet-Regi M. Biotinylation of silicon-doped hydroxyapatite: a new approach to protein fixation for bone tissue regeneration. Acta Biomater 2010;6:743-749.

30. Hing KA, Revell PA, Smith N, Buckland T. Effect of silicon level on rate, quality and progression of bone healing within silicate-substituted porous hydroxyapatite scaffolds. Biomaterials 2006;27:5014-5026.

31. Boanini E, Torricelli P, Gazzano M, Della Bella E, Fini M2, Bigi A. Combined effect of strontium and zoledronate on hydroxyapatite structure and bone cell responses. Biomaterials 2014;35:5619-5626.

32. Boyd AR, Rutledge L, Randolph LD, Meenan BJ. Strontium-substituted hydroxyapatite coatings deposited via a co-deposition sputter technique. Mater Sci Eng C Mater Biol Appl 2015;46:290-300.

33. Chung CJ, Long HY. Systematic strontium substitution in hydroxyapatite coatings on titanium via micro-arc treatment and their osteoblast/osteoclast responses. Acta Biomater 2011;7:4081-4087.

34. Tank KP, Chudasama KS, Thaker VS, Joshi MJ. Pure and zinc doped nano-hydroxyapatite: synthesis, characterization, antimicrobial and hemolytic studies. J Cryst Growth 2014;401:474-479.

35. Ashuri M, Moztarzadeh F, Nezafati N, Hamedani AA, Tahriri M. Development of a composite based on hydroxyapatite and magnesium and zinc-containing sol-gel-derived bioactive glass for bone substitute applications. Mater Sci Eng C Mater Biol Appl 2012;32:2330-2339.

36. Fox K, Tran PA, Tran N. Recent advances in research applications of nanophase hydroxyapatite. Chemphyschem 2012;13:2495-2506.

37. Zakaria SM, Sharif Zein SH, Othman MR, Yang F, Jansen JA. Nanophase hydroxyapatite as a biomaterial in advanced hard tissue engineering: a review. Tissue Eng Part B Rev 2013;19:431-441.

38. Jokic B, Stamenkovic I, Zrilic M, Obradovic-Djuricic K, Petrovic R, Janackovic D. Silicon-doped biphasic α-calcium-phosphate/hydroxyapatite scaffolds obtained by a replica foam method using uniform pre-annealed spherical particles. Mater Lett 2012;74:155-158.

39. Byun IS, Sarkar SK, Anirban Jyoti M, et al. Initial biocompatibility and enhanced osteoblast response of Si doping in a porous BCP bone graft substitute. J Mater Sci Mater Med 2010;21:1937-1947.

40. Kim YH, Jyoti MA, Youn MH, et al. In vitro and in vivo evaluation of a macro porous beta-TCP granule-shaped bone substitute fabricated by the fibrous monolithic process. Biomed Mater 2010;5:035007.

41. Kamitakahara M, Imai R, Ioku K. Preparation and evaluation of spherical Ca-deficient hydroxyapatite granules with controlled surface microstructure as drug carriers. Mater Sci Eng C Mater Biol Appl 2013;33:2446-2450.

42. Eshraghi S, Das S. Micromechanical finite-element modeling and experimental characterization of the compressive mechanical properties of polycaprolactone-hydroxyapatite composite scaffolds prepared by selective laser sintering for bone tissue engineering. Acta Biomater 2012;8:3138-3143.

43. Laschke MW, Strohe A, Menger MD, Alini M, Eglin D. In vitro and in vivo evaluation of a novel nanosize hydroxyapatite particles/poly(ester-urethane) composite scaffold for bone tissue engineering. Acta Biomater 2010;6:2020-2027.

44. Shin YM, Jo SY, Park JS, Gwon HJ, Jeong SI, Lim YM. Synergistic effect of dual-functionalized fibrous scaffold

with BCP and RGD containing peptide for improved osteogenic differentiation. Macromol Biosci 2014;14:1190-1198.

45. Nie L, Chen D, Yang Q, et al. Hydroxyapatite/poly-l-lactide nanocomposites coating improves the adherence and proliferation of human bone mesenchymal stem cells on porous biphasic calcium phosphate scaffolds. Mater Lett 2013;92:25-28.

46. Milovac D, Gamboa-Martinez TC, Ivankovic M, Gallego Ferrer G, Ivankovic H. PCL-coated hydroxyapatite scaffold derived from cuttlefish bone: in vitro cell culture studies. Mater Sci Eng C Mater Biol Appl 2014;42:264-272.

47. Gunn JM, Rekola J, Hirvonen J, Aho AJ. Comparison of the osteoconductive properties of three particulate bone fillers in a rabbit model: allograft, calcium carbonate (Biocoral®) and S53P4 bioactive glass. Acta Odontol Scand 2013;71:1238-1242.

48. Hench LL, Pantano CG Jr, Buscemi PJ, Greenspan DC. Analysis of bioglass fixation of hip prostheses. J Biomed Mater Res 1977;11:267-282.

49. Hench LL. Bioceramics. J Am Ceram Soc 1998;81:1705-1728.

50. Piotrowski G, Hench LL, Allen WC, Miller GJ. Mechanical studies of the bone bioglass interfacial bond. J Biomed Mater Res 1975;9:47-61.

51. Erol MM, Mourino V, Newby P, et al. Copper-releasing, boron-containing bioactive glass-based scaffolds coated with alginate for bone tissue engineering. Acta Biomater 2012;8:792-801.

52. Vrouwenvelder WC, Groot CG, de Groot K. Better histology and biochemistry for osteoblasts cultured on titanium-doped bioactive glass: bioglass 45S5 compared with iron-, titanium-, fluorine- and boron-containing bioactive glasses. Biomaterials 1994;15:97-106.

53. Khorami M, Hesaraki S, Behnamghader A, Nazarian H, Shahrabi S. In vitro bioactivity and biocompatibility of lithium substituted 45S5 bioglass. Mater Sci Eng C Mater Biol Appl 2011;31:1584-1592.

54. ElBatal HA, Khalil EM, Hamdy YM. In vitro behavior of bioactive phosphate glass–ceramics from the system P2O5–Na2O–CaO containing titania. Ceram Int 2009;35:1195-1204.

55. Cannas M, Indemini E, Krajewski A, Ravaglioli A, Contoli S. In vitro observations of iron-doped bioactive glasses. Biomaterials 1990;11:281-285.

56. Sabareeswaran A, Basu B, Shenoy SJ, Jaffer Z, Saha N, Stamboulis A. Early osseointegration of a strontium containing glass ceramic in a rabbit model. Biomaterials 2013;34:9278-9286.

57. Silva Lazaro G, Santos SC, Resende CX, Santos EA. Individual and combined effects of the elements Zn, Mg and Sr on the surface reactivity of a SiO2·CaO·Na2O·P2O5 bioglass system. J Non Cryst Solids 2014;386:19-28.

58. Vedel E, Zhang D, Arstila H, Hupa L, Hupa M. Predicting physical and chemical properties of bioactive glasses from chemical composition: part 4: tailoring compositions with desired properties. Glass Technol Eur J Glass Sci Technol A 2009;50:9-16.

59. Brink M. The influence of alkali and alkaline earths on the working range for bioactive glasses. J Biomed Mater Res 1997;36:109-117.

60. Elgayar I, Aliev AE, Boccaccini AR, Hill RG. Structural analysis of bioactive glasses. . J Non Cryst Solids 2005;351:173-183.

61. Bellucci D, Cannillo V, Sola A. Calcium and potassium addition to facilitate the sintering of bioactive glasses. Mater Lett 2011;65:1825-1827.

62. Sarkar SK, Sadiasa A, Lee BT. Synthesis of a novel bioactive glass using the ultrasonic energy assisted hydrothermal method and their biocompatibility evaluation. J Mater Res 2014;29:1781-1789.

63. Wu C, Chang J. Multifunctional mesoporous bioactive glasses for effective delivery of therapeutic ions and drug/growth factors. J Control Release 2014;193:282-295.

64. Zhang Y, Cheng N, Miron R, Shi B, Cheng X. Delivery of PDGF-B and BMP-7 by mesoporous bioglass/silk fibrin scaffolds for the repair of osteoporotic defects. Biomaterials 2012;33:6698-6708.

65. Wang X, Li X, Ito A, Sogo Y. Synthesis and characterization of hierarchically macroporous and mesoporous CaO-MO-SiO(2)-P(2)O(5) (M=Mg, Zn, Sr) bioactive glass scaffolds. Acta Biomater 2011;7:3638-3644.

66. Chen QZ, Thouas GA. Fabrication and characterization of sol-gel derived 45S5 Bioglass®-ceramic scaffolds. Acta Biomater 2011;7:3616-3626.

67. Sepulveda P, Jones JR, Hench LL. Bioactive sol-gel foams for tissue repair. J Biomed Mater Res 2002;59:340-348.

68. Niemela T, Niiranen H, Kellomaki M, Tormala P. Self-reinforced composites of bioabsorbable polymer and bioactive glass with different bioactive glass con-

tents. Part I: Initial mechanical properties and bioactiv-ity. Acta Biomater 2005;1:235-242.

69. Maquet V, Boccaccini AR, Pravata L, Notingher I, Jerome R. Porous poly(alpha-hydroxyacid)/Bioglass composite scaffolds for bone tissue engineering I: Preparation and in vitro characterisation. Biomaterials 2004;25:4185-4194.

70. Cattini A, Bellucci D, Sola A, Pawlowski L, Cannillo V. Suspension plasma spraying of optimised functionally graded coatings of bioactive glass/hydroxyapatite. Surf Coat Technol 2013;236:118-126.

71. Rojaee R, Fathi M, Raeissi K. Electrophoretic deposition of nano structured hydroxyapatite coating on AZ91 magnesium alloy implants with different surface treat-ments. Appl Surf Sci 2013;285:664-673.

72. Dorozhkin SV. Biocomposites and hybrid biomaterials based on calcium orthophosphates. Biomatter 2011;1:3-56.

73. Okamoto M, John B. Synthetic biopolymer nanocom-posites for tissue engineering scaffolds. Prog Polym Sci 2013;38:1487-1503.

74. Liu Y, Lim J, Teoh SH. Review: development of clinically relevant scaffolds for vascularised bone tissue engineer-ing. Biotechnol Adv 2013;31:688-705.

75. Swetha M, Sahithi K, Moorthi A, Srinivasan N, Rama-samy K, Selvamurugan N. Biocomposites containing natural polymers and hydroxyapatite for bone tissue engineering. Int J Biol Macromol 2010;47:1-4.

76. Sokolsky-Papkov M, Agashi K, Olaye A, Shakesheff K, Domb AJ. Polymer carriers for drug delivery in tissue engineering. Adv Drug Deliv Rev 2007;59:187-206.

77. Sahoo S, Ang LT, Goh JC, Toh SL. Growth factor de-livery through electrospun nanofibers in scaffolds for tissue engineering applications. J Biomed Mater Res A 2010;93:1539-1550.

78. Bao M, Lou X, Zhou Q, Dong W, Yuan H, Zhang Y. Electrospun biomimetic fibrous scaffold from shape memory polymer of PDLLA-co-TMC for bone tissue en-gineering. ACS Appl Mater Interfaces 2014;6:2611-2621.

79. Ribeiro N, Sousa SR, van Blitterswijk CA, Moroni L, Monteiro FJ. A biocomposite of collagen nanofibers and nanohydroxyapatite for bone regeneration. Biofabrica-tion 2014;6:035015.

80. Kim YH, Lee BT. Novel approach to the fabrication of an artificial small bone using a combination of sponge replica and electrospinning methods. Sci Technol Adv Mater 2011;12:035002.

81. Kim BR, Nguyen TB, Min YK, Lee BT. In vitro and in vivo studies of BMP-2-loaded PCL-gelatin-BCP electro-spun scaffolds. Tissue Eng Part A 2014;20:3279-3289.

82. Park JB. The use of hydrogels in bone-tissue engineer-ing. Med Oral Patol Oral Cir Bucal 2011;16:e115-e118.

83. Slaughter BV, Khurshid SS, Fisher OZ, Khademhosseini A, Peppas NA. Hydrogels in regenerative medicine. Adv Mater 2009;21:3307-3329.

84. Amirian J, Thuy Ba Linh N, Min YK, Lee BT. The effect of BMP-2 and VEGF loading of gelatin-pectin-BCP scaffolds to enhance osteoblast proliferation. J Appl Polym Sci 2014 Aug 11 [Epub]. http://dx.doi.org/10.1002/app.41241.

85. Nguyen MK, Alsberg E. Bioactive factor delivery strate-gies from engineered polymer hydrogels for therapeutic medicine. Prog Polym Sci 2014;39:1236-1265.

86. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Sci-ence 1999;284:143-147.

87. Montjovent MO, Burri N, Mark S, et al. Fetal bone cells for tissue engineering. Bone 2004;35:1323-1333.

88. Dee KC, Anderson TT, Bizios R. Osteoblast popula-tion migration characteristics on substrates modified with immobilized adhesive peptides. Biomaterials 1999;20:221-227.

89. Rezania A, Healy KE. Biomimetic peptide surfaces that regulate adhesion, spreading, cytoskeletal organization, and mineralization of the matrix deposited by osteo-blast-like cells. Biotechnol Prog 1999;15:19-32.

90. Burdick JA, Anseth KS. Photoencapsulation of osteo-blasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. Biomaterials 2002;23:4315-4323.

91. Weinand C, Pomerantseva I, Neville CM, et al. Hydro-gel-beta-TCP scaffolds and stem cells for tissue engi-neering bone. Bone 2006;38:555-563.

92. Lawson MA, Barralet JE, Wang L, Shelton RM, Triffitt JT. Adhesion and growth of bone marrow stromal cells on modified alginate hydrogels. Tissue Eng 2004;10:1480-1491.

93. Simmons CA, Alsberg E, Hsiong S, Kim WJ, Mooney DJ. Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by trans-planted bone marrow stromal cells. Bone 2004;35:562-569.

94. Schantz JT, Hutmacher DW, Lam CX, et al. Repair of

calvarial defects with customised tissue-engineered bone grafts II: evaluation of cellular efficiency and efficacy in vivo. Tissue Eng 2003;9 Suppl 1:S127-S139.

95. Fowler EB, Cuenin MF, Hokett SD, et al. Evaluation of pluronic polyols as carriers for grafting materials: study in rat calvaria defects. J Periodontol 2002;73:191-197.

96. Nath SD, Abueva C, Kim B, Lee BT. Chitosan-hyaluronic acid polyelectrolyte complex scaffold crosslinked with genipin for immobilization and controlled release of BMP-2. Carbohydr Polym 2015;115:160-169.

97. Barbetta A, Rizzitelli G, Bedini R, Peccib R, Dentini M. Porous gelatin hydrogels by gas-in-liquid foam templating. Soft Matter 2010;6:1785-1792.

98. Nam YS, Park TG. Biodegradable polymeric microcellular foams by modified thermally induced phase separation method. Biomaterials 1999;20:1783-1790.

99. Wen Y, Grondahl L, Gallego MR, Jorgensen L, Moller EH, Nielsen HM. Delivery of dermatan sulfate from polyelectrolyte complex-containing alginate composite microspheres for tissue regeneration. Biomacromolecules 2012;13:905-917.

100. Wan AC, Tai BC, Schumacher KM, Schumacher A, Chin SY, Ying JY. Polyelectrolyte complex membranes for specific cell adhesion. Langmuir 2008;24:2611-2617.

101. Boddohi S, Moore N, Johnson PA, Kipper MJ. Polysaccharide-based polyelectrolyte complex nanoparticles from chitosan, heparin, and hyaluronan. Biomacromolecules 2009;10:1402-1409.

102. Wan AC, Yim EK, Liao IC, Le Visage C, Leong KW. Encapsulation of biologics in self-assembled fibers as biostructural units for tissue engineering. J Biomed Mater Res A 2004;71:586-595.

103. Shovsky A, Varga I, Makuska R, Claesson PM. Formation and stability of water-soluble, molecular polyelectrolyte complexes: effects of charge density, mixing ratio, and polyelectrolyte concentration. Langmuir 2009;25:6113-6121.

104. Shovsky A, Bijelic G, Varga I, Makuska R, Claesson PM. Adsorption characteristics of stoichiometric and nonstoichiometric molecular polyelectrolyte complexes on silicon oxynitride surfaces. Langmuir 2011;27:1044-1050.

105. Abbah SA, Liu J, Lam RW, Goh JC, Wong HK. In vivo bioactivity of rhBMP-2 delivered with novel polyelectrolyte complexation shells assembled on an alginate microbead core template. J Control Release 2012;162:364-

372.

106. Chu H, Johnson NR, Mason NS, Wang Y. A [polycation:heparin] complex releases growth factors with enhanced bioactivity. J Control Release 2011;150:157-163.

107. Czichocki G, Dautzenberg H, Capan E, Vorlop KD. New and effective entrapment of polyelectrolyte-enzyme-complexes in LentiKats. Biotechnol Lett 2001;23:1303-1307.

108. Trubetskoy VS, Loomis A, Hagstrom JE, Budker VG, Wolff JA. Layer-by-layer deposition of oppositely charged polyelectrolytes on the surface of condensed DNA particles. Nucleic Acids Res 1999;27:3090-3095.

109. Liu W, Sun S, Cao Z, et al. An investigation on the physicochemical properties of chitosan/DNA polyelectrolyte complexes. Biomaterials 2005;26:2705-2711.

110. Nguyen TB, Lee BT. A combination of biphasic calcium phosphate scaffold with hyaluronic acid-gelatin hydrogel as a new tool for bone regeneration. Tissue Eng Part A 2014;20:1993-2004.

111. Hench LL. Bioceramics: from concept to clinic. J Am Ceram Soc 1991;74:1487-1510.

112. Tancred DC, Carr AJ, McCormack BA. The sintering and mechanical behavior of hydroxyapatite with bioglass additions. J Mater Sci Mater Med 2001;12:81-93.

113. Suchanek W, Yashima M, Kakihana M, Yoshimura M. Processing and mechanical properties of hydroxyapatite reinforced with hydroxyapatite whiskers. Biomaterials 1996;17:1715-1723.

114. Jun YK, Kim WH, Kweon OK, Hong SH. The fabrication and biochemical evaluation of alumina reinforced calcium phosphate porous implants. Biomaterials 2003;24:3731-3739.

115. Hu H, Xu G, Zan Q, et al. In situ formation of nano-hydroxyapatite whisker reinfoced porous β-TCP scaffolds. Microelectron Eng 2012;98:566-569.

116. Kang Y, Scully A, Young DA, et al. Enhanced mechanical performance and biological evaluation of a PLGA coated β-TCP composite scaffold for load-bearing applications. Eur Polym J 2011;47:1569-1577.

117. Lee BT, Sarkar SK, Song HY. Novel bamboo-like fibrous, micro-channeled and functional gradient microstructure control of ceramics. Mater Trans 2008;49:339-344.

118. Feng P, Wei P, Li P, Gao C, Shuai C, Peng S. Calcium silicate ceramic scaffolds toughened with hydroxyapatite whiskers for bone tissue engineering. Mater Charact

2014;97:47-56.

119. McNamara SL, Rnjak-Kovacina J, Schmidt DF, Lo TJ, Kaplan DL. Silk as a biocohesive sacrificial binder in the fabrication of hydroxyapatite load bearing scaffolds. Biomaterials 2014;35:6941-6953.

120. Giannitelli SM, Accoto D, Trombetta M, Rainer A. Current trends in the design of scaffolds for computer-aided tissue engineering. Acta Biomater 2014;10:580-594.

121. Melchels FP, Domingos MA, Klein TJ, Malda J, Bartolo PJ, Hutmacher DW. Additive manufacturing of tissues and organs. Prog Polym Sci 2012;37:1079-1104.

122. Bartolo PJ, Almeida H, Laoui T. Rapid prototyping and manufacturing for tissue engineering scaffolds. Int J Comput Appl Technol 2009;36:1-9.

123. Peltola SM, Melchels FP, Grijpma DW, Kellomaki M. A review of rapid prototyping techniques for tissue engineering purposes. Ann Med 2008;40:268-280.

124. Liu X, Rahaman MN, Fu Q. Bone regeneration in strong porous bioactive glass (13-93) scaffolds with an oriented microstructure implanted in rat calvarial defects. Acta Biomater 2013;9:4889-4898.

125. Rubshtein AP, Trakhtenberg ISh, Makarova EB, et al. Porous material based on spongy titanium granules: structure, mechanical properties, and osseointegration. Mater Sci Eng C Mater Biol Appl 2014;35:363-369.

126. Kim YH, Jyoti MA, Youn MH, et al. In vitro and in vivo evaluation of a macro porous beta-TCP granule-shaped bone substitute fabricated by the fibrous monolithic process. Biomed Mater 2010;5:035007.

127. Gomez-Barrena E, Rosset P, Lozano D, Stanovici J, Ermthaller C, Gerbhard F. Bone fracture healing: cell therapy in delayed unions and nonunions. Bone 2015;70:93-101.

128. Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920-926.

129. Kaigler D, Pagni G, Park CH, et al. Stem cell therapy for craniofacial bone regeneration: a randomized, controlled feasibility trial. Cell Transplant 2013;22:767-777.

130. Jager M, Hernigou P, Zilkens C, et al. Cell therapy in bone healing disorders. Orthop Rev 2010;2:e20.

131. Hernigou P, Mathieu G, Poignard A, Manicom O, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions: surgical technique. J Bone Joint Surg Am 2006;88 Suppl 1:322-327.

132. Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. J Bone Joint Surg Am 2005;87:1430-1437.