Chapter 1

Fabrication Methodologies of Biomimetic and Bioactive Scaffolds for Tissue Engineering Applications

Mythili Prakasam, Madalina Popescu, Roxana Piticescu and Alain Largeteau

Additional information is available at the end of the chapter

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Abstract

Tissue engineering has offered wide technologies for developing functional biomaterials substitutes for repair and regeneration of damaged tissue and organs. Biomimetic materials with their inherent nature to mimic natural materials are possible through the recent advances in the fabrication technology. With the help of porous or dense implants made with biodegradable materials, it is possible to incorporate different vital growth factors, genes, drugs, stem cells and proteins. In this review, we presented various fabrication methodologies of biomimetic and bioactive scaffolds for tissue engineering applications. An overview of the nanocomposites of biomaterials is presented. Further an example of one of the hybrid nanocomposite material is given for additive manufacturing.

Keywords: biomimetic scaffolds, bioactive ceramics, fabrication technology, tissue engineering, 3D printing

1. Introduction

Inspired by the natural evolution mechanisms and their structural, physical and chemical characteristics of biological organisms, researchers worldwide are looking for synthetic materials that mimic the function of the natural materials. Nature offers solutions to many complex technological and materials solutions due to the billon of years of technological evolution. Biomimetics [1] is considered as a future of materials design and productions. The history of biomimetics exploration by humans dates back to early fifteenth century by Leonardo da Vinci speculating the clues of possibility of human air travel following the mechanics of flight of birds. Biomimetic materials have potential applications in various domains such as biology, chemistry, materials science and electronics. In the present scenario, the need for synthetic bone

substitutes [2] in the last few decades have increased owing to the number of accidents/trauma and inherent bone defects. The current state-of-art enables the successful fabrication of the materials [3] for biomimicking applications especially for biological applications. Mimicking the 3D structure and extracellular matrix (ECM) [4] of native tissue is critical for successful cell transplantation and growth of artificial tissue. Biomimetic scaffolds are therefore necessary to recapitulate this natural environment and provide various cues to direct cell processes and differentiation. For examples, when considering a biomaterial to be used in implants or bone grafts, various aspects such as biocompatibility, osteogenic properties, bioactivity and its mechanical functions, based on its functionalities have to be studied. To replicate an ideal scaffold as biomimetic for tissue applications, fabrication methodologies play a very major role. Especially, tissue engineering has a great promise in using the biomimetic technologies for organ transplantation [5], reconstructive surgery [6] and artificial extracellular matrices [7] for three dimensional tissue formation. In the field of tissue engineering various approaches are followed such as cell substitution for specified functions, delivery of induced substances such as cell growth and differentiation and growth of cells in three dimensional scaffolds. Bone reconstruction is clinically important due to the large number of patients who have bone defects of critical size, leading to decrease in overall health and quality of life. The main treatment for bone defects remains bone grafting [8]. Approximately, 600,000 bone graft procedures are performed each year in the United States, and about 2.2 million of such procedures are performed worldwide annually, generating a sale of about 2.5 billion dollars per year and representing the second most common tissue transplantation procedure after blood [9–13].

There are a variety of scaffold fabrication methods in tissue engineering, including gas foaming, solvent casting and particulate leaching, emulsion freeze-drying, phase separation, melt molding, membrane lamination, electrospinning, fiber bonding, polymer/ceramic composite foam fabrication or a combination of these techniques [9, 14–16]. In order to replicate the natural bones, scaffolds are engineered to be bioactive or bioresorbable to enhance tissue growth. Scaffolds are often porous that supports mechanically. There are various criteria such as biocompatibility, mechanical properties, pore size and bioresorbability that have to be considered as requirements of an ideal scaffold. Different classes of materials are used for fabrication of scaffolds such as polymers, bioglass, composite and metals. Significant progress was achieved in terms of scaffolds for mechanical support with better osteogenesis and angiogenesis. Various material fabrication methodologies enable the possibility to fabricate scaffolds with complex design [17] to ensure mechanical integrity and scaffold interconnectivity. The objective of this chapter is to review the different fabrication methodologies and the recent advances in the fabrication of biomimetic and bioactive scaffolds are presented with a case study and the possible improvements envisaged are discussed.

2. Biomimetic nanocomposites types and fabrication methodologies

2.1. Various biomimetic nanocomposites based on their design

Human body being a complex and sensitive biological system requires the scaffold materials with diverse and challenging characteristics. Scaffold must combine structural, material,

bioactivation, signaling molecules, cells and biological requirements satisfied for different applications. Human bone [18] is classified as the long bones (femur and tibia), short bones (vertebrae and metacarpal bone), flat bones and the spongious bone [19]. The structural property of the corresponding bones is strongly interdependent on the mechanical property [20]. The cortical bone contains 10% porosity and is mostly dense, whereas the spongy bones are highly porous and has less mechanical property. The spongy bones act as a host to soft tissues, cartilage, and meniscus and prevent the stress concentration. The fibrous tissue surrounding the bones is ligaments, which are highly organized fiber tissue composed of collagens, elastin, proteoglycan, water and cells. The mineral inorganic part will be assisting in compression and shear and the collagen matrix provides tensile strength. Anisotropic compact bone has compression strength in the range of 130–225 MPa along the longitudinal direction and has the compression strength in the range of 105–135 MPa in transverse direction [21]. The cancellous/spongy bone has compression strength of 5–10 MPa and elastic modulus of 50–100 MPa. In the current state of art, it can be observed that the mechanical integrity of the synthetic scaffolds is inadequate. Polymers have yielded results close to the cancellous bone properties. Bimodal architectures [22] matching the natural bone is yet in progress. Scaffolds should provide sufficient strength [23] for cell ingrowth in *in vitro* conditions and integrate completely under *in vivo* conditions. In order to fabricate the biomimetic materials various systems [24] such as polysaccharides, proteins, nanocomposites based on calcium phosphates (CaPs) (e.g., β-tricalcium phosphate, hydroxyapatite) with good osteoconductivity, resorbability and biocompatibility are employed. The polymers [25] employed usually assist in structural integrity and promotes cell adhesion. Whereas, CaPs are biocompatible, osteoconductive and biodegradable with limitations on mechanical strength that forbids the load-bearing applications. β-TCP is the high temperature phase of CaPs, but has the chemical similarities with HAp, with different resorbing capability [26]. Resorption of HAp is slow in the biological medium and β-TCP has good reabsorption. Ionic co-substitutions in calcium phosphates are also used that can influence the structure, microstructure, crystallinity and dissolution rate. Various ions [27] such as Sodium, Strontium, Magnesium, Manganese, Silver, Barium Potassium, Zinc, Fluoride, Chlorides and Carbonates are substituted in hydroxyapatite. The substitution of these aforesaid ions have significant role in causing alterations to bone resorption, bone formation, solubility, structure and morphological changes and enhanced surface microstructure. There are also Calcium phosphate based cements [28] that can be used as injectable pastes in the defect bone site for cell delivery and is an upsurging topic of research. Nanocomposites [29] consisting the component of polymer and nanosized CaPs improves the tissue bonding, cell adhesion and cell differentiation. Designing of the 3D scaffold [30] should stimulate the adhesion, proliferation and differentiation mechanisms in addition to the structural complexity of natural bones. The synthetic bone should also enable vascularization to enable biointegration with transport and support of signaling molecules, passage of nutrients and blood vessels. Hence the critical aspects such as highly porous interconnected microstructure and degree of porosity for uniform cell distribution, proliferation and migration *in vitro*. Critical pore sizes [31] are necessary to adjudge their function, because when the pore size is small the cell adherence can block the pores and matrix formation of the scaffold. An optimal pore size of ~150–400 μm can help in bone tissue regeneration. On the other hand, the high porous scaffold will have low Young's modulus and compressive strength. It was reported that the tangent elastic modulus of natural bones decreases with the increase in porosity such as in porous tantalum [32]. The Young's modulus

value of wet compact bone is in the range of 6–11 GPa, a porous scaffold of Ni-Ti with porosity in the range of 20–50 Vol % was fabricated by powder metallurgy.

Equally the pore shape, size and orientation are important in adjudging the mechanical properties of the scaffolds. Variation of the mechanical properties [33] of the scaffold with two different pore structures was reported earlier. It was observed that the mechanical properties of the scaffold with spherical pore structure have higher elastic modulus in comparison to the scaffold with cylindrical pore [34]. Similar alteration in the mechanical properties was also reported in the case of polycaprolactone [35]. From the aforesaid it can be concluded that the mechanical properties of the fabricated scaffold can be altered by the variation of the pore shape. Similar influence was reported on the pore size, where an increase in the pore size reduces the mechanical properties. The orientation of the pores also significantly altered the mechanical properties of the scaffold irrespective of the matrix of the material chosen ranging from metals to polymers. When the pore geometry was parallel or aligned in the same direction, the mechanical strength of these scaffolds were high. The mechanical stability of the multiscale porosity can be interesting in terms of crack propagation. This multiscale porosity can be achieved by combining two or three scaffold fabrication methods.

2.2. Materials for fabrication of scaffolds

Materials for fabrication of scaffolds [36] are selected based on their degradability, chemical and physical properties. Materials can be classified based on their source and mode of origin/preparation. Animal and plant based materials such as starch, alginate, chitosan, hyaluronic acid, gelatin, collagen, fibrin, silk, etc., are used as natural biomaterials. Due to the disease transmission and purification, synthetic biomaterials are attracting the interest of the researchers. Synthetic organic and inorganic materials such as HAp, CaPs, glass, polyesters are actively studied for usage as scaffold material. Autograft bone scaffold [37] was considered to the gold standard for bone repair defect. Lack of donors and possible disease infection are few of the major disadvantages of autograft scaffolds. Bone allograft [38] was considered as an alternative to the autograft, but still the transmission of diseases, inflammatory reactions and rejection by the recipient body is prevalent. Hence artificial scaffolds have been proposed as an alternative to the autograft and allograft scaffolds. Usage of metallic implants [39] dates back to 1920. Currently synthetic bone implants made of metals, ceramics, composites and glasses are employed for bone regeneration and bone reconstruction. There are several issues such as poor strength of the polymer scaffolds, poor ion release of ions from the metallic scaffolds, brittleness of the ceramics and difficulty in controlling degradation rate of the composite scaffold to be addressed. Langer and Vacanti [40] were the pioneers of the tissue engineering concept in early nineties. They introduced the concept of introducing the bone marrow cells, different growth factors, gene and drug delivery in to the artificial bone scaffolds. Further investigations by various researchers enabled the possibility of improving the biocompatibility by changes in the topology, nanostructure, chemistry and structural aspects of the scaffolds. Metallic biomaterials are especially preferred for their usage in load-bearing applications due to their good mechanical properties. The porous metallic scaffolds [41] based on the stainless steel, titanium and magnesium are used to eliminate stress shielding and reduce the stiffness to match the natural bone. Interconnected porous metallic scaffolds [42]

were fabricated by combining the various rapid prototype techniques such as solvent leaching and 3D printing was reported in the fabrication of Ti scaffolds. Other fabrication techniques such as powder metallurgy, sintering and rapid prototyping to modulate the scaffold design. The mechanical properties of the Ti scaffolds can be used to mimic the human cancellous bone due to its structural flexibility. The elasticity and other vital properties such as deformation force, stress and strain can be modified by alloying with a suitable metal. NiTi alloys were reported to exhibit good *in vivo* compatibility than pure Ti porous scaffolds [43]. The porous metallic scaffold assisted in rapid formation of new bone tissues for load-bearing conditions.

Bioactive glasses [44] were reported to have good osteoconductivity, controlled biodegradability, cell delivery capabilities and inducing osteogenic gene expression for formation of bone minerals and capability for drug delivery. But bioactive glasses yield poor results for load-bearing applications due to their lack of superelastic performance, mismatch of compressive strength and Young's modulus of the natural bone. In order to improve the mechanical properties of the bioactive glasses, modification of structure and composition is achieved during bioglass scaffold fabrication. Biomorphic and mesoporous bioactive scaffolds [45] were shown to have better mechanical properties than in comparison to the classical bioactive glasses. The inorganic component phase in the bioactive glass scaffold is important in addition to the structural design of these bioactive glass scaffolds. Biopolymers and its derived composites are also used for fabrication of scaffold for tissue engineering applications. However their poor mechanical strength makes it difficult to use them in load bearing applications. Biopolymer based composites and hybrids for bone scaffold applications [46] with required strength are prepared by varying the volume fraction of polymer in the composites. ECM structure [47] provides the structural and biochemical support, hence ECM like biopolymers, ECM-HAp composites and ECM bioglass scaffolds are studied for tissue engineering applications. In order to replicate the biomimetic conditions, surface modifications are also carried out. Controlled release of biological molecules is also a key function that the scaffolds play.

2.3. Biomimetic porous scaffolds

Porous biomimetic scaffolds [48] with their 3D structure is advantageous for (1) better cell biomaterial interactions, cell adhesion and growth (2) interconnected porosity for angiogenesis and transport of nutrients, regulatory factors for cell survival, proliferation and differentiation (3) Sufficient structural integration with good tensile strength and elasticity (4) Control degradation and minimal toxicity *in vivo*. Nanocomposites of CaPs and natural polymers such as collagen and gelatin are well known for tissue engineering. Techniques such as foam replica method [49], freeze casting [50], freeze drying [51], phase separation [52], gas foaming [53], rapid prototyping [54] and electrospinning [55] are employed for fabrication. The challenge of nanocomposite scaffolds lies in the ensuring the retention of chemical phases and retaining the porous structure without disturbing their porous structure. A combination of different method of fabrications such as freeze casting and electrospinning are used for fabrication of scaffolds. Other polymers such as poly lactic co-glycolic acid (PLGA), Sodium dodecyl sulfate (SDS), cellulose, poly glycolic acid (PGA), poly (ethylene glycol) and poly l-lactic acid (PLA) are also used for fabricating the nanocomposites due to their excellent biodegradability and biocompatibility. Collagen of type I, the predominantly available protein in mammals can be

readily obtained from animal tissues and from human tissues. This extracellular matrix protein collagen can be reconstituted in a different morphology into fibrillary matrix by changing the pH and temperature of precursors. Due to the lack of mechanical strength in collagen for *in vivo* applications, several strategies are employed such as crosslinking with hydrogels [56] or compression so that it can sustain or resist cell-induced contraction. Inherent characteristics of collagen such as dipole moment and alignment under strong magnetic field [57] were demonstrated to induce cell migration and allow preferential growth of neurites along the alignment of the fibril direction. Collagens can hold several cellular receptors that can variate the cell behavior and their biological function can be induced by combining with growth factors for example, vascular endothelial growth factor (VEGF) to improve cardiac function was observed.

Fibrin [58] is a specialized protein network clinically available from autologous sources such as human blood plasma. In the presence of thrombin protease, fibrin matrix is formed spontaneously by polymerization of fibrinogen. Cell migration in fibrin is dependent on the cell-associated proteolytic activity from plasmin and MMPs due to their small diameter. This indeed assists in strong fibril-fibril interaction and the natural network formation and stabilization through covalent bond stabilization. As fibrin matrices are poorly active for most of the cell types, functionalization with ECM peptides or growth factors is necessary. The controlled release of the growth factors and hormones can be efficiently done by covalent bonding of biomolecules. This possibility of delayed/controlled release of the growth factors/hormones [59] is currently employed for clinical evaluation for local bone repair.

Hyaluronan or Hyaluronic acid [60] is a structural protein that is noncovalently attached to the protein core and entwine ECM. Due to their strong anionic nature, these polymers absorb water and hence providing the compressive strength to the ECM. Various chemical hyaluronic acid derivatives have been prepared by controlling the functional group and the type of covalent bond. It is possible to create a wide range of materials with diverse properties. Hyaluronic acid [61] is used for various applications for dermal wound healing, chondrocyte transplantation for tissue repair and for incorporation of other functional biomolecules for improved fibroblast proliferation and wound healing. Other self-assembling polypeptides are also used to form nanofibrillar matrices *in situ*. Self-assembled peptide hydrogels [62] are used as a tool for developing 3D cell culture plates.

To have properties similar to natural ECM, it is necessary to have facilities for cell seeding, adhesion, proliferation, differentiation and new tissue generation. Essential characteristics such as biodegradability and mechanical properties are important to be studied. The biodegradation of the scaffold should be in coherence with the rate of the formation of the new tissue formation that it supports initially to act as a scaffold material to serve as a template. Elastomeric properties of scaffolds [63] are studied to improve their applications in tissue engineering applications. Other than the natural ECM materials, synthetic polymers such as poly (ε-caprolactone) (PCL) and polyurethanes (PU) [64] are investigated for vascular and other tissue engineering applications. Tensile modulus and strength are critical parameters necessary for tendons and ligaments. Natural fibrous protein from silk worm cocoon is a

material with excellent tensile and mechanical strength [65]. Though Sericin present in the natural silk causes adverse immune responses and is disadvantageous for tissue engineering applications, natural silk's biocompatibility is improved upon Sericin removal. Silk fibroins have hydrophobic and hydrophilic blocks which forms crystals through hydrophobic interactions and hydrogen bonding resulting in the improved tensile strength. Spider silk fibroin polymers [66] are used for genetic engineering due to their excellent mechanical and cell adhering capacity.

As extracellular proteins have a fibrous structure with diameters in the nanometer or submicrometer scales, various advanced material shaping techniques were developed. Techniques such as electrospinning, self-assembly and phase separation are a few worthy-to-mention. Electro-spinning technique were used to produce nanofibers, but the disadvantage of this technique is to fabricate the complex 3D scaffold structure or to produce intricate pore structures. Various cells were reported to proliferate, differentiate and attach on these electro-spun nanofibers [67]. Phase separation arises when homogeneous multicomponent system tends to be unstable resulting in multiphase system due to the system free energy. This technique is employed in generating porous structures as tissue engineering scaffolds. The physical form of the porosity (closed/open) depends on the system of phase separation. The huge disadvantage of this technique is the lack of interconnected porosity and the lack of control over 3D shapes. Biological effects of nanofibrous scaffold [68] were found to adsorb more human serum proteins in comparison to the scaffolds of smooth pore wall morphology. The cell adhesion proteins were comparatively well detected than the conventional scaffolds.

Hydroxyapatite being the major inorganic compound used in most of the composite scaffolds, it provides good osteoconductivity. In the presence of the composite structures with polymers hydroxyapatite provides structure and design flexibility. Further it assists in improving the protein adsorption capacity, microstructure favoring the bone tissue regeneration and diminishing the cell death. These nanocomposites mimic the features similar to the natural bone with improved mechanical properties. However regarding the biodegradation rate of stoichiometric HAp are less efficient than the partially carbonated apatite. The surface of scaffolds affect cellular response and in turn also the tissue regeneration [69].

2.4. Biomimetic scaffold fabrication

Composites of scaffold materials with organic and inorganic components were designed for mechanical and physiological requirements in tissue engineering. However the brittle nature of ceramics and low mechanical strength inhibits their usage in clinical applications. Composite structure of HAp with porous polymers alongside their pore size, shape and morphology has improved the mechanical strength for tissue engineering applications. With the recent advances in the biodegradable polymers [70], glass and ceramics, it is possible to cater degradation kinetics and resorption *in vivo* after stimulating cellular responses at the molecular level. It is expected that the new generation of scaffolds can perfectly mimic the natural bone in terms of mechanical and structural aspects. With the advances in the manufacturing techniques such as selective laser sintering and rapid prototyping should

be helpful in bone tissue engineering applications. These techniques are widely used in the fabrication of temporomandibular joints [71], craniofacial [72] or periodontal structures [73]. The fabrication methodology allows the flexibility of the combination of different materials for increasing mechanical strength. The current state-of-art, however, does not allow to exactly replicate the natural architecture of the extracellular matrix or the natural bone. There is a continuous demand for the improvement and functioning of the scaffold fabrication. Here we will discuss few of the fabrication techniques such as solvent casting, particulate leaching electrospinning, gas foaming, phase separation, fiber meshing and bonding, self-assembly, rapid prototyping, melt molding, lamination and freeze drying.

2.4.1. Solvent casting

A nineteenth century technology, which was initially used for production of thin films, is currently employed for diverse applications ranging from optical applications, photographical film base flexible printed circuits and high temperature resistive films. Solvent casting [74] are generally produced by evaporation of the solvent in order to form the scaffolds. In order to successfully employ the solvent casting, it is necessary that the polymer used should be soluble in a volatile solvent or in water. A stable solution with an adequate solid content and viscosity should be formed. Formation of homogeneous film after removing from cast support must be possible. The main disadvantage of this technique is the denaturation of the protein caused due to the solvent if toxic and influence of the organic material on the solvent. To remove the toxicity of the solvent left over in the scaffold, they are usually dried by vacuum process and dried completely. To make it time efficient, other techniques such as particulate leaching were combined for the fabrication of scaffolds.

2.4.2. Particulate-leaching technique

Being one of the popular techniques in the fabrication of scaffolds for tissue engineering [75] is relatively a technique of ease. Usage of porogens such as salt, sugar and wax is common to produce the pore channels by this technique. Mixture of porogen of the desired size is mixed with the material, then after leaching the porogen, the pores are left behind in the matrix. The control of the pore size is possible by choosing the required size and shape of the porogen. Pore sizes in the range of 300–500 μm is possible with a porosity percentage of around 94–95%, however the control of the interconnected pore is not possible. Thin layers of up to 3 mm thickness is feasible with this technique.

2.4.3. Gas foaming

Gas foaming process uses the utilization of high pressure gases for the fabrication of highly porous scaffolds [53]. While the polymers are used, they get saturated and gets nucleated as a gas bubble with the gas bubble size ranging in the order of 100–500 μm are used. When the gas is injected the gas bubble starts to create the phase separation. A three dimensional porous structure can be obtained by using the gas foaming technique. But the control over the interconnectivity is highly lagging behind in this technique, though is advantageous in terms as a solvent free technique. The porosity will be frequently absent on the external surface.

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2.4.4. Phase separation

This technique involves quenching of the polymers which can cause two phases of polymers to separate as polymer rich and polymer poor phases [76]. The polymer poor phase will cause the porous structure network to be formed. The control of the porous microstructure is possible with the help of parameters such as polymer concentration, quenching rate and temperature, solvent concentration, solvent type and dispersion of the solute molecules. The solvent can be removed by extraction, evaporation and sublimation after integrating the bioactive molecules in the scaffold. Nanofibers can be prepared by liquid-liquid phase separation to replicate the architecture similar to type I collagen molecules and other extracellular matrix architectures for 3D cell culture environment. As these experiments are carried out at low temperatures, the incorporation of bioactive molecules is feasible. The phase separation methodology can be used in conjunction with other techniques that can control the porous architecture and also with other rapid prototyping techniques for tissue engineering applications.

2.4.5. Sintering/heat molding

This technique [77] involves filling the mold with the mixture of powder/polymer and porogen and heating/sintering above the glass transition temperature of the polymer or at the porogen evaporation or melting temperature. The external shaping of the sample will take form of the die set up/mold used for sintering or heat molding. When the porogen is leached out and then the scaffold is formed. The inconvenience of this technique is that the possibility of residual porogen and impossibility to incorporate biological molecules due to the involvement of high temperature processing.

2.4.6. Freeze casting

This technique [78] involves the mixing the solvent and the solute followed by decrease of temperature until to reach negative temperature to fabricate ice crystals. The solute molecules are segregated around the ice crystals forming a porous network. In order to remove the solvent, lyophilization is carried out which involves applying lower pressure than the equilibrium vapor phase of the frozen solvent. After sublimation of the solvent ice crystals, the porous network remains intact. The beneficial factors of this technique is the possibility of changing the morphology of the pores by modulating the temperature of ice crystals and the possibility to incorporate bioactive molecules due to the low processing temperature involved in this fabrication methodology. The pore size distribution and the homogeneity of the porosity formed can be controlled with the aid of the processing temperature of the process. Freeze casting can also be used to protect other dry biological samples to retain their bioactivity.

2.4.7. Fiber Mesh

This technique [79] involves interweaving or weaving individual fibers into a three dimensional scaffold with varying pore size. This technique enable the availability of the cell attachment and allows transport of the nutrient for the cell survival and cell growth. Though the practical

difficulties such as lack of structural stability exists. In order to increase the structural stability and increase the crystallinity heat treatment is sometimes employed to overcome the structural instability in Fiber Mesh technique.

2.4.8. Fiber bonding

This technique [80] involves combining the nonfiber polymer mixing with a solvent, when the solvent is removed then the solute molecules will be embedded in the polymer matrix. The scaffolds are fabricated by bonding the collagen matrix to polymers with threaded collagen fibers. The fiber bonding encapsulation takes place due to the heat treatment. The resulting scaffold structure has large surface area that allows the cell proliferation by replicating the extracellular matrix. The disadvantage of this technique involves the poor controlling ability of the porosity and the pore size, unavailability of suitable solvents and appropriate melting temperature of the polymers.

2.4.9. Electrospinning

This technique [81] is highly used for fabricating the nanofibrous architecture. Nanofibrous architecture highly favorises cell binding and other cell behavior activities. Electrospinning was successfully employed in fabricating porous scaffolds of nano- and microscale fibers that can replicate the structural and biological functions of the natural extracellular matrix. The method involves electrostatic spraying of polymer coatings. Scaffolds with >90% were obtained by this methodology. An appropriate solvent is required for dissolving the polymer to be loaded in a syringe. The rate of control of polymer flow can be adjusted by constant syringe flow rate. Various parameters are necessary for scaffold fabrication by electrospinning such as viscosity, conductivity, molecular weight and surface tension of the polymers and the processing parameters such as applied voltage, flow rate and temperature. The main advantage of this technique is that it can produce 3D scaffold configuration adaptable for cell and tissue organization, adhesion and spatial cell regeneration with suitable physiological conditions. The main disadvantage of this technique is the impossibility of cell seeding, but cryospinning and templated sacrificial scaffold can create the pore of desired size in the electrospun matrices. Electrospinning is also used in conjunction with other scaffold techniques such as solvent casting and rapid prototyping techniques. Favorable results were observed in cell culture for cartilage and bone tissue engineering applications with electrospun scaffolds.

2.4.10. Rapid prototyping

The group of techniques [82] involving the assistance of computer assisted design (CAD) and computer assisted manufacturing (CAM) such as stereolithography, selective laser sintering, fused deposition modeling, three dimensional printing and three dimensional plotting. The choice of materials ranges from polymers, ceramics to metals. These fabrication techniques are frequently used for fabrication of biomimetic tissue scaffolds. Most of these techniques are capable of yielding scaffolds with good mechanical strength, high accuracy and possibility of incorporation of cells and proteins. There are few limitations such as elevated temperatures, weak bonding

between the powder particles, trapped powders in the pores (requiring post treatment measures) and slow fabrication processing in few cases. These materials are prevalently employed in bone, heart valves, adipose tissues and cartilages. Hybrid materials based on calcium phosphates with polymers is also widely used with rapid prototyping also known by terminology as additive manufacturing. In the following section, we will focus on one of the widely used rapid prototyping technique called as 3D printing with an elaborate example as a case study.

2.5. Hybrid materials based on calcium phosphates and polyurethanes for bone tissue engineering – a case study

Additive manufacturing (AM), also known as rapid prototyping or solid freeform fabrication, was developed in the mid-1980s as an advanced technology to overcome the limitations of these conventional methods and have received much attention in recent years due to its ability to deliver a high level of control over the architecture of the construct [13]. With AM techniques, scaffolds with precise geometries can be prepared, using computer-aided design combined with medical imaging techniques (either computed tomography – CT, scanning or magnetic resonance imaging – MRI) to make anatomically shaped implants [83].

According to ISO/ASTM52900-15, there are seven categories of ALM type processes: Binder Jetting, Directed Energy Deposition, Material Extrusion, Material Jetting, Powder Bed Fusion, Sheet Lamination and Vat Photopolymerization [84]. AM techniques may be divided into three sub-groups: (1) laser or other directed energy beam, (2) print or "ink," and (3) nozzle systems [85]. C.K. Chua and W. Y. Yeong have classified AM techniques for the fabrication of tissue scaffolds in two types of methods: (1) direct methods and (2) indirect methods [16], summarized in **Table 1**.

Several Rapid Prototyping (RP) techniques, such as selective laser sintering (SLS), fused deposition modeling (FDM), precision extrusion deposition (PED) and 3-D fiber deposition (3DF) have been used to fabricate polymer–calcium phosphate composites based on poly(hydroxybutyrate-co-hydroxyvalerate), PLLA, PCL or poly(ethylene oxide terephthalate)/poly(butylene terephtalate) (PEOT/PBT) co-polymer and different calcium phosphates, such as carbonated hydroxyapatite, HA or β-tricalcium phosphate (β-TCP) [85].

In 2014, Zhou et al. [86] proposed a new method to increase the printability of calcium phopshate powders, by mixing it with $CaSO_4$ (HA: $CaSO_4$ = 25:75 weight ratio) for scaffold fabrication through 3D printing method. 3D printing technique has been used for fabrication of 3D scaffolds based on hydroxyapatite (HA) and organic polymers such as polyvinyl alcohol (PVA) [87] or collagen [88].

A scaffold made of composite material based on calcium phosphate and collagen was obtained by 3D printing method at low temperatures. For this purpose, 5–20% phosphoric acid was used as binder and Tween 80 was added as a non-cytotoxic surfactant in the proportion of 0.25% in the binder solution. The optimal concentration of the binder solution (8.75%) for which the cytocompatibility and mechanical strength are maximized has been established. Collagen has previously been dissolved in the binder solution to further enhance the fabrication of calcium phosphate-collagen composites by 3D printing.

Type of method		
Direct		**Indirect**
Melt-dissolution deposition techniques	**Particle bonding techniques**	
Fused deposition modeling (FDM)	Selective laser sintering (SLS)	Droplet deposition
Precision extruding deposition (PED)	Three dimensional printing (3D printing) or color jet printing (CJP)	Melt deposition
3D fiber deposition technique (3DF)	TheriForm	Photo-polymerization: – stereolithography – two-photon polymerization
Precise extrusion manufacturing (PEM)		
Low-temperature deposition manufacturing (LDM)		
Multi-nozzle deposition manufacturing (MDM)		
Robocasting		
Pressure-assisted microsyringe (PAM)		
3D bioplotter		
Rapid prototyping robotic dispensing system (RPBOD)		

Table 1. Classification of additive manufacturing (AM) techniques for scaffold fabrication.

In 2013, Nandakumar et al. [88] reported the production of two types of polymer-hydroxyapatite composite scaffold by 3D deposition using a Bioplotter. PolyActive ™ (PA), a commercial copolymer of poly (ethylene oxide-terephthalate)/poly (butylene terephthalate) (PEO/PBT) was used.

In the first embodiment, polymer-ceramic composite filaments with the desired mass ratio (maximum 15% HA) were extruded and further used for scaffold fabrication using a Bioplotter. In the second approach, polymer scaffolds were obtained by 3D deposition, while the ceramic particles were made in the form of columns by sintering the ceramic paste through stereolithography. The two components were then manually assembled by pressing HAp into the pores of the polymer scaffold, thus creating the composite material.

Among 3D printing techniques, *bioplotting* is the most used method in printing of 3D tissue constructs. Bioplotting, first developed in 2000 at the Freiburg Research Centre, is an extrusion-based printing method that is spatially controlled by a robotic system (x, y, and z directions) with microscale resolution, which uses STL files to guide the extrusion head. Nozzles of various diameters are used for dispensing highly viscous or pasty materials applying different actuation mechanisms – mainly screw – or piston based mechanical and pneumatic. The dispensing head moves in three dimensions, while the fabrication platform is stationary. The printed construct from the bioplotter occurs in a laminar fashion by the computer-controlled deposition of material on a surface and could be relatively large (several centimeters in length, width, and height) [82, 89].

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Since heating is not required, the system can process thermally sensitive biocomponents, and even cells. The strand thickness can be modulated by varying material viscosity, deposition speed (speed in the planar field), tip diameter, or the applied pressure. The main advantages of bioprinting using a Bioplotter system are the room temperature processing, direct incorporation of cells, and homogenous distribution of cells. The main disadvantages are limited mechanical stiffness, critical time of hardening, specific matching of material, and low resolution [90].

2.5.1. Fabrication of 3D scaffolds based on hydroxyapatite-polyurethane hybrids

Hydroxyapatite—polyurethane 3D scaffolds have been obtained using system BioScaffolder SysEng, Germany, (**Figure 1**) connected to a computer on which the Bioscaffolder SW version 3.0 software is installed.

HAp-PU nanostructured hybrid powders synthesized in high pressure hydrothermal conditions (1000 bar and 100 bar, respectively) were mechanically mixed with a carefully selected commercial polymer (Mowiflex, Kuraray Poval) 20% solution, and a crosslinking agent (Baymedix® FD103, Covestro), to obtain HAp-PU-based pastes used as a feedstock in the 3D Bioprinting technique. Some examples of 3D hybrid structures obtained with different nozzles and rotation angle between 2 successive layers (90° and 45°, respectively) are given in **Figure 2**.

2.6. Hydrothermal synthesis of hybrid organic-inorganic nanostructures in high pressure conditions

Hydrothermal method is a well-known and attractive technique for producing pure nanocrystalline, highly homogeneous nanoparticles in a single step, in aqueous medium, with low energy consumption [91–94]. The hydrothermal method has proven to be an effective, convenient and environmentally friendly process. Hydrothermal synthesis at high pressure is characterized by three main advantages: (i) low energy consumption, developed by applying

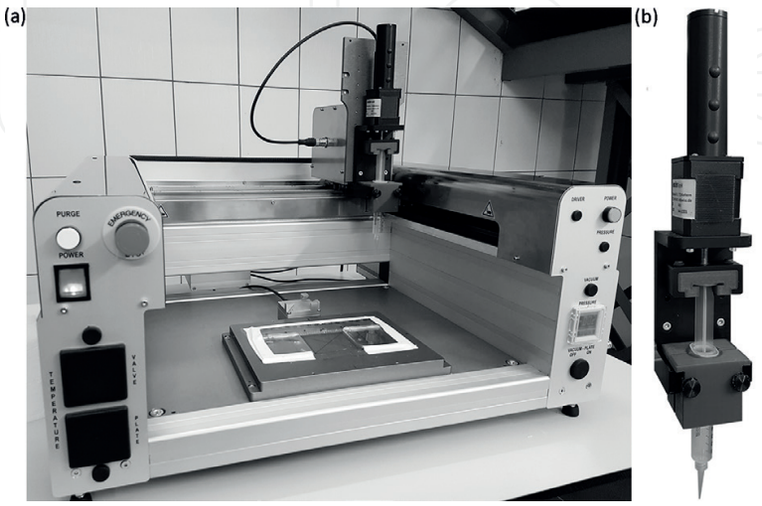

Figure 1. (a) System BioScaffolder, SYSENG and (b) low temperature deposition head.

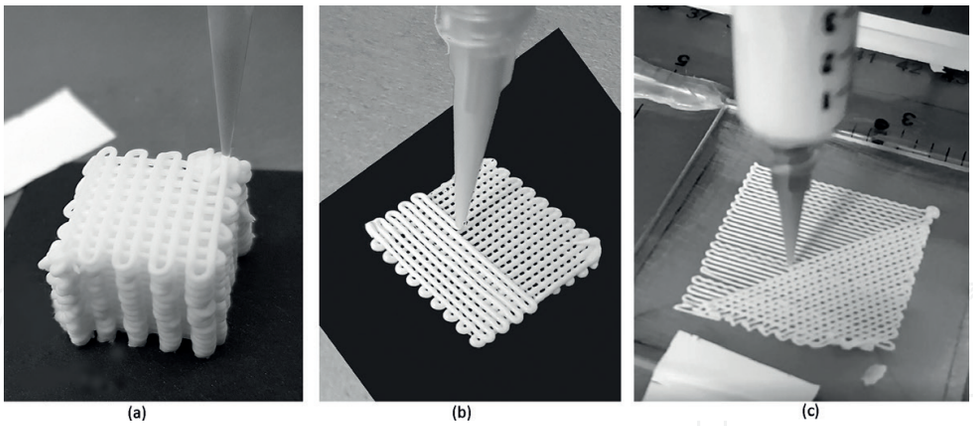

Figure 2. 3D printed scaffolds using three types of nozzles: (a) Ø 0.8 mm; (b) Ø 0.6 mm; and (c) Ø 0.4 mm [95].

pressure (for a liquid phase, the same energy is involved on five units for the temperature scale than on 4000 units for the pressure scale); (ii) negative ΔV value between the total molar volume of reaction products and the total molar volume of reactants and (iii) improvement of the chemical reactivity.

2.6.1. Hydrothermal synthesis at 100 bar using Ar gas

In a first step, $Ca(NO_3)_2$*$4H_2O$ (Sigma Aldrich) was dissolved in distilled water. Ammonium dihydrogen phosphate (Sigma Aldrich) was added dropwise into the calcium nitrate solution under continuous stirring. In parallel, the viscous polyurethane-diol solution was dissolved in water, and thus obtained solution was transferred into the initial vessel along with the other reagents. A precipitate was obtained whose pH was adjusted to 10 using 25% ammonia solution. In the second step, the hybrid precursor thus resulted was transferred to the Berghof autoclave, Germany and subjected to hydrothermal treatment at a pressure of 100 bar and a temperature of below 120°C for 3 hours. Pressure is created above the solution vessel of the autoclave using an argon gas bottle [93, 94]. After hydrothermal treatment, the nanostructured powder was washed with distilled water until neutral pH was reached, mixed with polyvinyl alcohol (PVA) 5% solution and spray dried in LabPLANT, United Kingdom spray dryer.

2.6.2. Hydrothermal synthesis at 1000 bar isostatic pressure

In a first step, $Ca(NO_3)_2$*$4H_2O$ (Sigma Aldrich) was dissolved in distilled water. Ammonium dihydrogen phosphate (Sigma Aldrich) was added dropwise into the calcium nitrate solution under continuous stirring. In parallel, the viscous polyurethane-diol solution was dissolved in water, and thus obtained solution was transferred into the initial vessel along with the other reagents. A precipitate was obtained whose pH was adjusted to 10 using 25% ammonia solution. In the second step, the hybrid precursor thus resulted was transferred to the high pressure autoclave HP Systems, France and subjected to hydrothermal treatment in isostatic pressure conditions (1000 bar) and a temperature below 120°C, for 3 hours. Isostatic pressure is created uniformly inside the autoclave by a hydraulic system.

The reaction mixture is perfectly sealed in a special synthesis bag, leading to formation of nanohybrids in microbiologically safe conditions.

After hydrothermal treatment, the nanostructured hybrid powder was washed with distilled water to a pH of 7, mixed with polyvinyl alcohol (PVA) 5% solution and spray dried in LabPLANT, United Kingdom spray dryer.

2.7. Structural characterization

2.7.1. Chemical analysis

Nanostructured hydroxyapatite-polyurethane hybrid powders were characterized by chemical quantitative analysis. Ca content was determined using Flame Atomic Absorption Spectrometry (FAAS) method. P content was measured using *Inductively Coupled Plasma Optical Emission Spectrometry* (ICP-OES) method according to ASTM E 1479/2011.

Ca and P content of some investigated hybrid samples is presented in **Table 2**.

Results presented in **Table 2** confirmed the formation of hydroxyapatite as the major phase of nanostructured powders, namely: Ca:P molar ratio is between 1.54 and 1.75, while in HPU-4 and HPU-8 samples is equal to theoretical value from pure hydroxyapatite.

2.7.2. FT-IR analysis

Powders characterization using Fourier transform infrared spectroscopy (FT-IR) revealed the presence of the following vibration bands: (i) the OH stretching vibration at 3550 cm^{-1} (sharp band) in the HA sample. In the HA-PU hybrid sample the intensity of this band is highly diminished; (ii) stretching vibration of the water molecule (3300 cm^{-1}). It is a broad band, seen in both the HA sample and the hybrid sample; (iii) the deformation vibration of the –OH group (1630 cm^{-1}) from HA, which completely disappears in the case of HA-PU sample. This, correlated with the decrease of the OH band at 3350 cm^{-1}, could be due to the interaction of the two components through the hydroxyl group; (iv) stretching vibrations of $(PO_4)_3$-group at 1094,

Sample name	Sample type	Ca, %	P, %	Ca:P molar ratio (calculated)
HPU-1	80% HA-20% PU	32.9	16.2	1.58
HPU-2	80% HA-20% PU	35.6	17.4	1.59
HPU-4	80% HA-20% PU	35.3	16.5	**1.66**
HPU-5	50% HA-50% PU	35.2	17.8	1.54
HPU-6	80% HA-20% PU	28	12.5	1.75
HPU-7	50% HA-50% PU	38.4	17.1	1.74
HPU-8	80% HA-20% PU	37	17.1	**1.67**

Note: The bold values represents the experimental molar ratio equivalent to theoretical values hydroxyapatite.

Table 2. Chemical analysis results.

1040 and 962 cm^{-1}, being characteristic for hydroxyapatite spectrum (IR fingerprint), can also be observed in the hybrid spectrum. The FT-IR spectra of the analyzed powders (hydroxyapatite, respectively hydroxyapatite-polyurethane based hybrids) are shown in **Figure 3**.

2.7.3. Thermal analysis (DSC-TG)

Powders characterization using thermal analysis (DSC-TG) revealed the presence of several endothermic peaks, as shown in **Table 3**. **Figures 4** and **5** show thermal analyses of HA and HA hybrid samples. Peak 1 could be attributed to the elimination of surface adsorbed water. Peak 2, observed only for HPU hybrid powder, could correspond to the removal of OH groups from water of constitution. Peak 3 in the hybrid sample, although characterized by a very low enthalpy (0.251 J/g), could be associated with breakage of polyurethane chain links. Peak 4 (466°C) of the HPU sample corresponds to the decomposition of soft polyurethane segments. The last two peaks of the hybrid sample are due to the burning of the organic phase. The total mass loss is 14.5%, which means that the polyurethane from the composition of HPU sample has almost completely decomposed. Endothermic peaks at 276 and 336°C observed in the case of hydroxyapatite (HA sample) can be explained by decomposing of the polyvinyl alcohol traces left after drying the sample in the spray dryer. The weight loss for the HA sample is 8.5%.

2.7.4. Particle size distribution by DLS technique

Particle size distribution determination with Malvern Zetasizer ZS90 granulometer is based on a non-invasive dynamic light scattering (DLS) emitted by a laser. The particle assimilated with a sphere is constantly displaced by Brownian motion as a result of statistical collisions with the liquid molecules. In this movement, small particles will move faster than large particles.

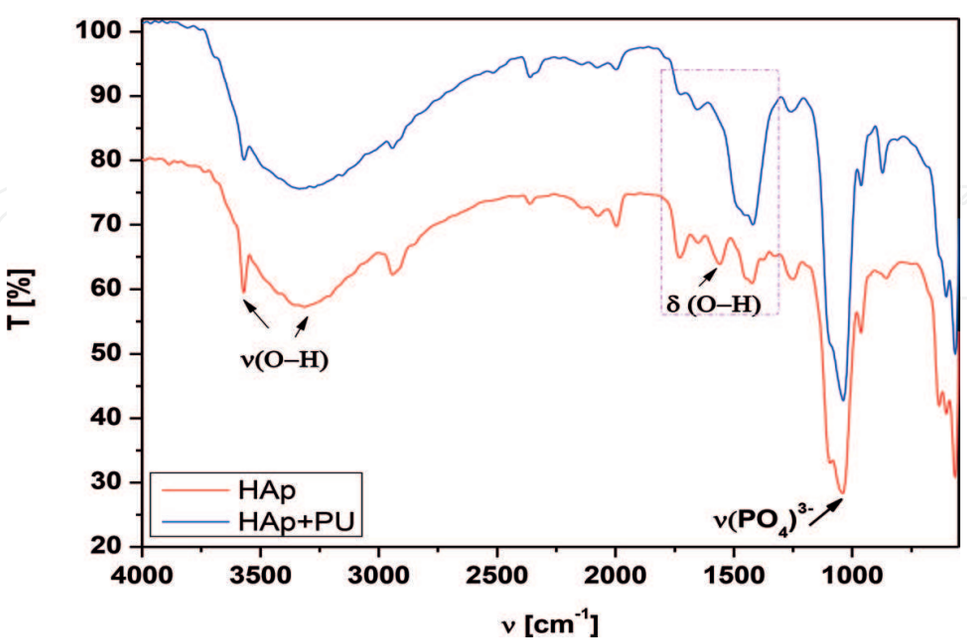

Figure 3. FT-IR spectra of one hybrid sample compared to pure hydroxyapatite.

Sample name	Peak 1 (endothermic)		Peak 2 (endothermic)		Peak 3 (endothermic)		Peak 4 (endothermic)		Peak 5 (endothermic)		Peak 6 (endothermic)	
	T, °C	ΔH, J/g	T, °C	ΔH, J/g	T, °C	ΔH, J/g	T, °C	ΔH, J/g	T, °C	ΔH, J/g	T, °C	ΔH, J/g
HA	58.1	32.019	–	–	276.1	11.963	336.4	21.498	–	–	825.6	13.61
HPU	63.5	49.549	135.8	4.429	246.2	0.251	466.6	4.746	711.4	6.226	784	38.762

Table 3. Results obtained from thermal analysis (DSC-TG).

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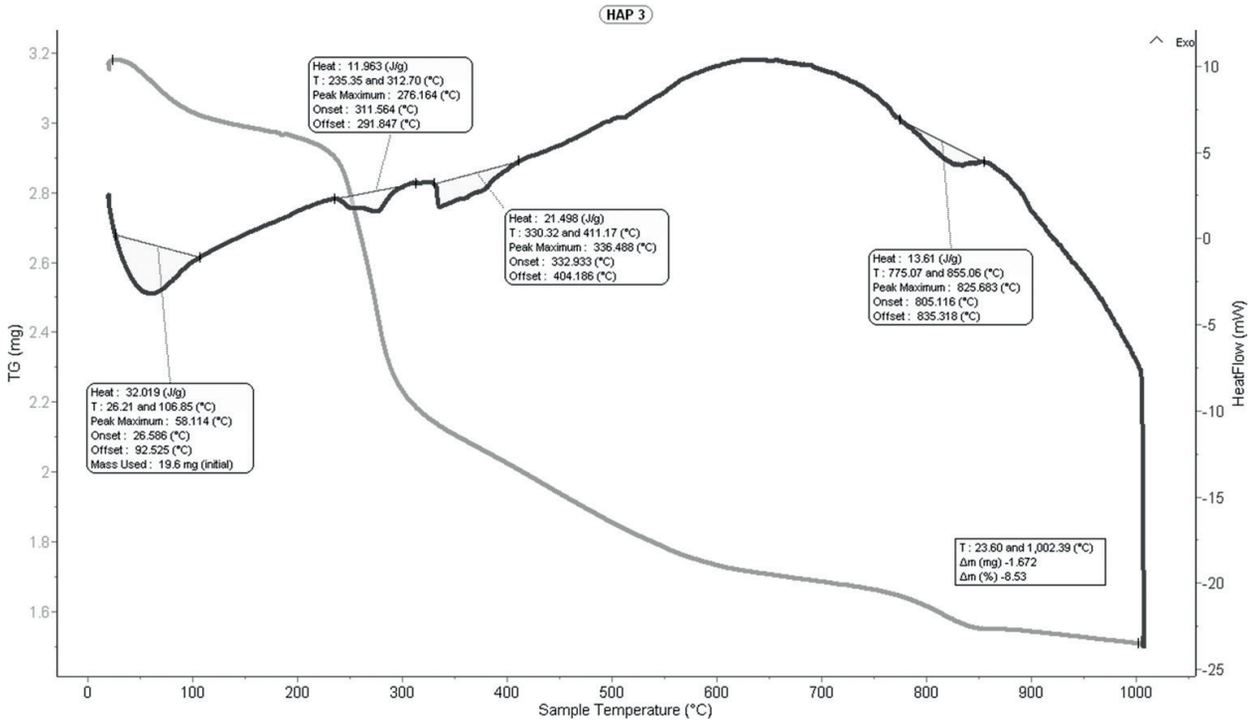

Figure 4. DSC graph and mass loss of pure hydroxyapatite.

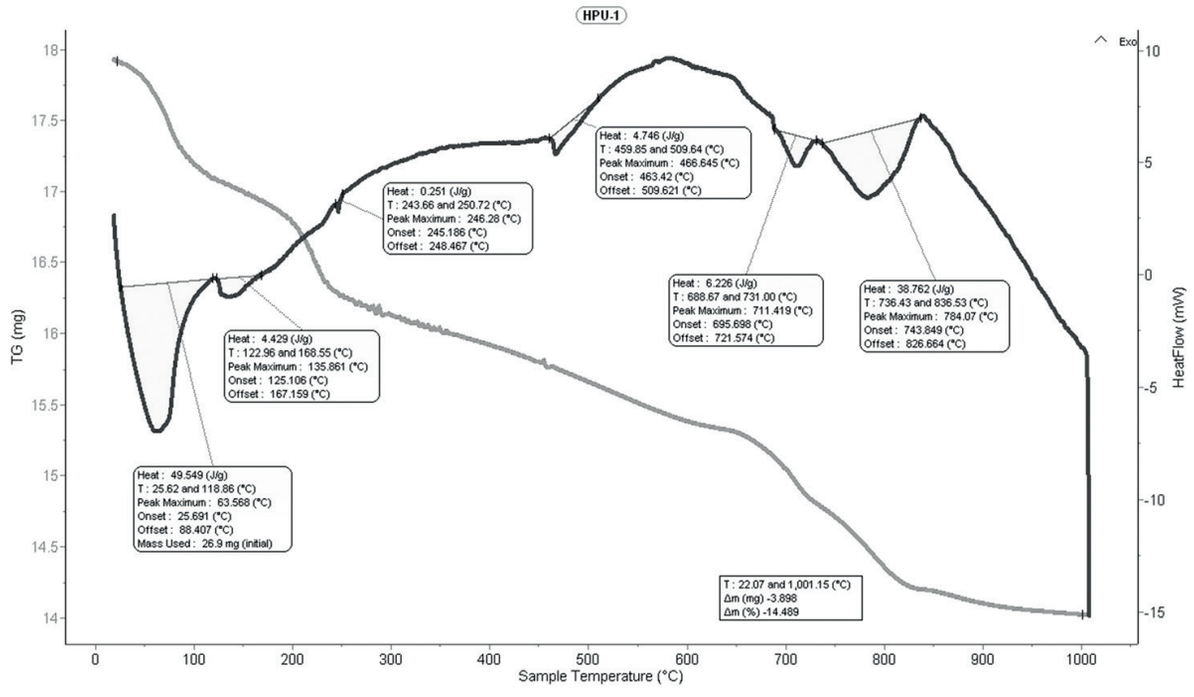

Figure 5. DSC-TG graph and mass loss of one hybrid sample.

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The particle size distribution obtained by DLS is a function of the relative intensity of light scattered by particles of different dimensional classes. The time dependence of the intensity fluctuations for the determination of the translational diffusion coefficient (D), as well as the hydrodynamic diameter (D_H) are measured by using equation (1). η is the viscosity of the investigated suspension.

$$D_H = \frac{kT}{3\pi\eta D} \quad (1)$$

The result is a distribution of intensity.

Sample preparation: stable, aqueous dispersions with known optical properties (required for size measurement) are prepared. Results are presented in **Table 4** and **Figure 6**.

It can be observed a decrease in the mean particle size of the hybrid material compared to pure hydroxyapatite, both for the filtered and the initial samples.

There is a slight tendency for agglomeration of the hybrid material (**Figure 6**).

Sample name	Mean particle size [nm]	Polydispersity index	Observations
HA	167.5	0.133	
HA filtered	146.3	0.061	Sample filtered through Millipore membrane (d = 0.22 μm)
HPU	119.7	0.238	
HPU filtered	64.65	0.229	Sample filtered through Millipore membrane (d = 0.22 μm)

Table 4. DLS analysis results.

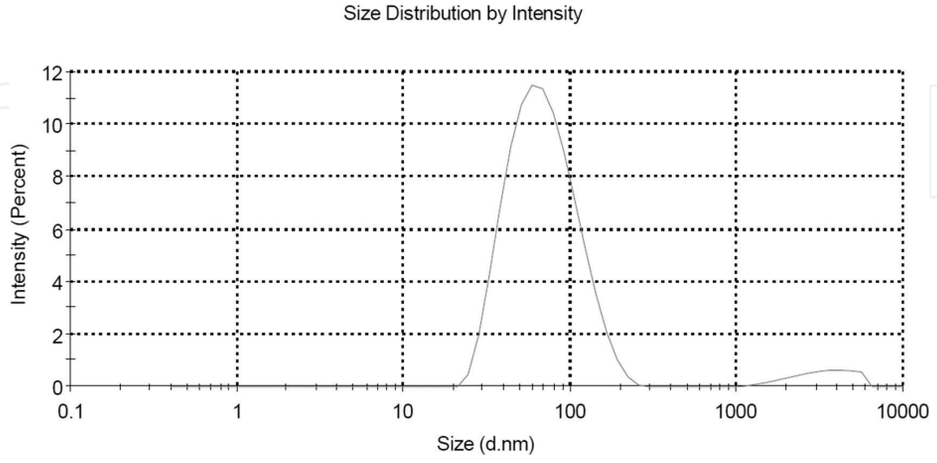

Figure 6. Particle size distribution of one hybrid sample filtered through 0.22 μm Millipore membrane.

2.7.5. HRTEM analysis

HRTEM analysis was performed with a High Resolution Transmission Electron Microscope (HRTEM) Tecnai F30, G2S Twin (1 Å line resolution) – FEI Company.

It can be observed rod like structures of hydroxyapatite inside hybrid structure, typical for hydrothermally prepared HA, as well diffraction lines which confirms sample crystallinity (**Figure 7**).

EDX spectrum (**Figure 8**) reveals Ca, P, O and C content of the investigated sample, in accordance with chemical analysis results. Moreover, it confirms the presence of organic phase (polyurethane), through C and O content.

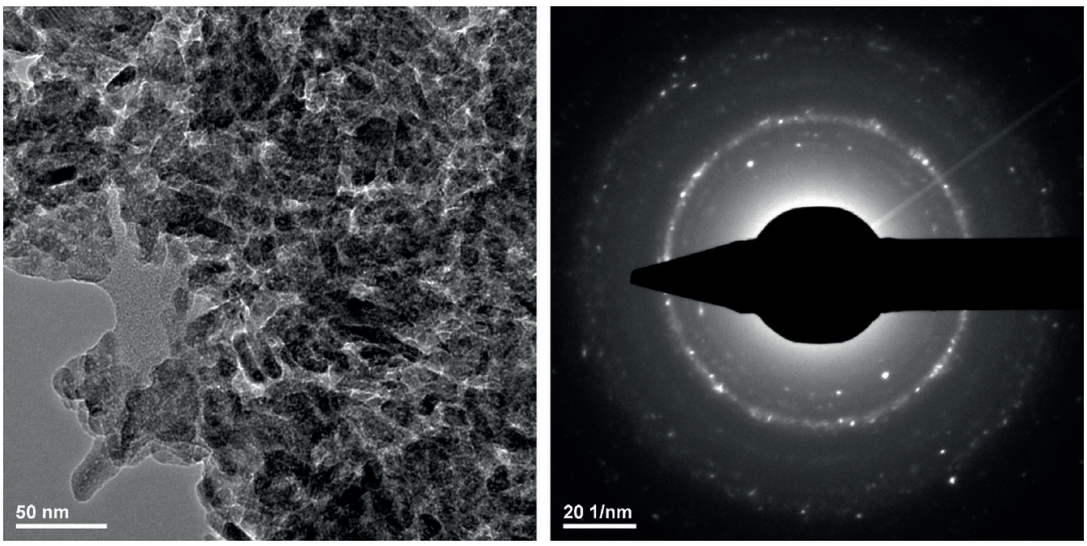

Figure 7. TEM images for one hybrid sample with 80% HA and 20% PU.

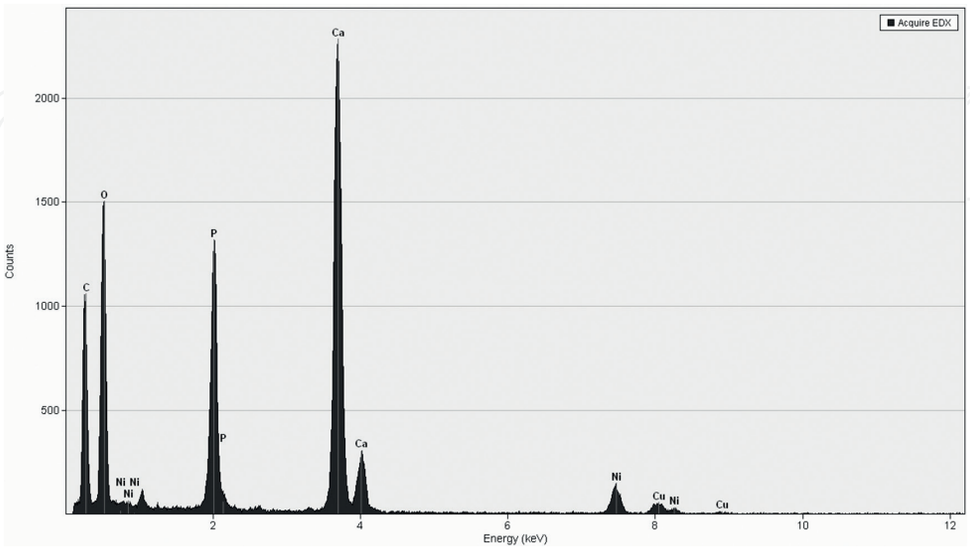

Figure 8. EDX spectra of one hybrid sample with 80% HA and 20% PU.

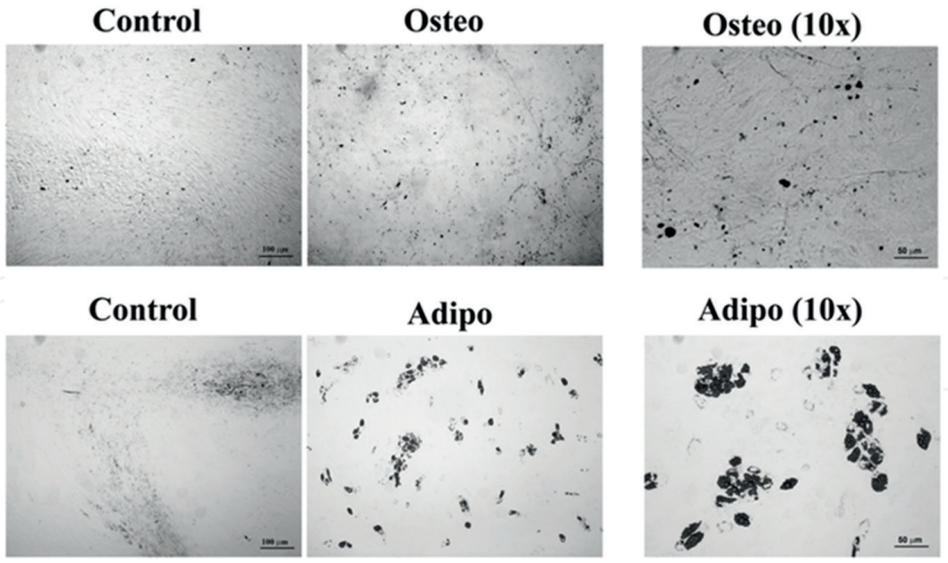

Figure 9. Optical microscopy illustrating the ability of human MSC to generate osteoblasts (up) and adipocytes (down) *in vitro*. UP: (left) Human MSC cultivated in normal growth medium (DMEM + 10% SBF-MSC qualified) and von Kossa stained; (Middle and right) human MSCs increased 2 weeks in osteogenic differentiation environment; The formation of aggregates strongly colored with silver nitrate is observed (von Kossa method). Down: (left) Human MSC cultured in normal growth medium (DMEM + 10% SBF-MSC qualifies) and stained with Oil Red O; (Middle and right) human MSCs increased 2 weeks in adipogenic differentiation environment; There is evidence of accumulation of red-colored lipid charges with Oil Red O.

2.8. Biocompatible properties

MSC cells were characterized in terms of their multipotent potential, demonstrating their ability to differentiate *in vitro* into adipocytes and osteoblasts (**Figure 9**). Dilutions tested were: 1/10, 1/20, 1/50, 1/100 and 1/200. Diluted solutions were added to both cultured fresh cells and after they were maintained at 37°C under 5% CO_2 (pH equilibration). Also, for each condition, cell interaction studies were performed in culture, both 24 hours after cell adhesion and 48 hours after cell adhesion (assuming that in the first case we can see the effect of HAp on cellular proliferation, and in the latter case, HAp effect on cell viability).

3. Conclusion

The current chapter deals with the various fabrication methodologies of biomimetic and bioactive scaffolds that can be employed for the tissue engineering applications. Biomimetic nanocomposites scaffolds have the ability to mimic the structural and mechanical properties of native tissues. Various fabrication methodologies are available to replicate and produce the biomimetic biomaterials for tissue engineering applications. Various advantages and disadvantages of these fabrication methodologies are discussed here. Different classes of materials are used for biomimetic applications and designing of these biomimetic materials are cited with examples. Recent advances and research on scaffolds with controlled nano/micro architecture, pore distribution and pore density reveal the potential of the nanocomposites

for tissue engineering applications. Technologies such as additive manufacturing, electrospinning and other combined recent advanced manufacturing technologies holds promise for new advances in the field of tissue engineering. Due to space constraints the present chapter has given one of the examples of the possibility to fabricate biomimetic materials by additive manufacturing. Further developments are required in the field of biomaterials fabrication to exactly replicate the biomimetic conditions.

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Author details

Mythili Prakasam[1]*, Madalina Popescu[2], Roxana Piticescu[2] and Alain Largeteau[1]

*Address all correspondence to: mythili.prakasam@icmcb.cnrs.fr

1 CNRS, Université de Bordeaux, ICMCB, Pessac, France

2 National R&D Institute for Nonferrous and Rare Metals, Pantelimon, Romania

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