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Hydroxyapatite as a Biomaterial to Improve Stem Cell-Related Tissue Engineering

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ABSTRACT

Abbreviations: ESCs: Embryonic Stem Cells; SSEA: Stage-Specific Embryonic Antigen; ASC: Adult Stem Cells; MSCs: Mesenchymal Stem Cells; PLA: Polylactic Acid; HA: Hydroxyapatite; PU: Poly-Ester Urethane; BSA: Bovine Serum Albumin; FCS: Fetal Calf Serum; OC: osteocalcin; CAD: Computer aided design; PCL: Polycaprolactone

Introduction

The optimum goal in periodontics is to prevent periodontal diseases as a major aim, control and regenerate lost tissues in the periodontal apparatus when it takes place. The optimum goal of full regeneration of periodontal structure was always difficult to achieve due to complexity of the periodontal apparatus, which suffers a slow rate of regeneration abilities; on the other hand, this does not mean that there is no regenerative ability of the periodontium [1]. As a result of these facts, the theory of guided tissue regeneration was first postulated by Melcher [2], who pointed to the importance of hindering the growth of unwanted cell lines from the healing areas to allow the growth of the desirable cell lines [2]. As a result of the positive outcome of the *in vitro* and *in vivo* studies of regeneration in the periodontal research in the 80s of the last century, this theory has been challenged in dental therapy, which entailed different outcomes, drawbacks and even complications, which caused different uses of techniques and materials according to the different anatomical and pathological conditions of the clinical cases. There is a growing need for bone regeneration due to various clinical bone diseases, such as bone infections, bone tumors and bone loss by trauma [3]. Current therapies for bone defects include autografts, allografts, xenografts and other artificial substitutes, such as metals, synthetic cements and bioceramics [4,5]. However, these substitutes are far from ideal and each has specific problems and limitations (Table 1). For example, autografts are associated with donor shortage and donor site morbidity, whereas allografts and xenografts have the risk of disease transmission, immune response [6] and synthetic materials wear not resembling natural bone.

Table 1: Different bone graft materials and their limitations.

Bone Graft Type	Limitations
Autograft	Additional surgical site, with other site morbidity, pain and possible complications
Allograft	Disease transmission, immune reaction, ethical issues.
Xenograft	High infection risk, different biological behavior, lack of heterogeneity, different microenvironment with possibility of immune response
Alloplast	Heterogeneity and immune reaction, potential harboring of infectious agents, different mechanical properties than natural bone, different mineral content.

Autograft has osteoconduction and osteoinduction abilities, besides its osteogenic potential, which means can deposit new bone by its native cells, but its limitations are additional surgical site, with other site morbidity, pain and possible complications [7]. Regenerative medicine and tissue engineering used interchangeably, while in fact tissue engineering is a field of regenerative medicine, which does not encompassing it totally. Tissue engineering is a field of research applying engineering principles on biological sciences

in the goal of restoring and maintaining lost tissue function or even improves existing function [8]. On the other hand, a sufficient definition of regenerative medicine is to replace human cells or tissues in the goal of restoring total or partial loss of organ function, which includes the use cell and/or gene therapy [9] (Figure 1). In summary we have to say that although guided tissue regeneration provided a strong basis of periodontal tissue regeneration and not just healing, but the limitation of the availability of bone graft material that fulfill the essential needs of tissue regeneration created the need for tissue engineering science, aiming for having optimal results by changing the architecture of the scaffolds, optimizing its biocompitability and physical properties, while regarding the biologics by using the science of genetics, stem cell biology, and enhancing the knowledge of biological growth factors.

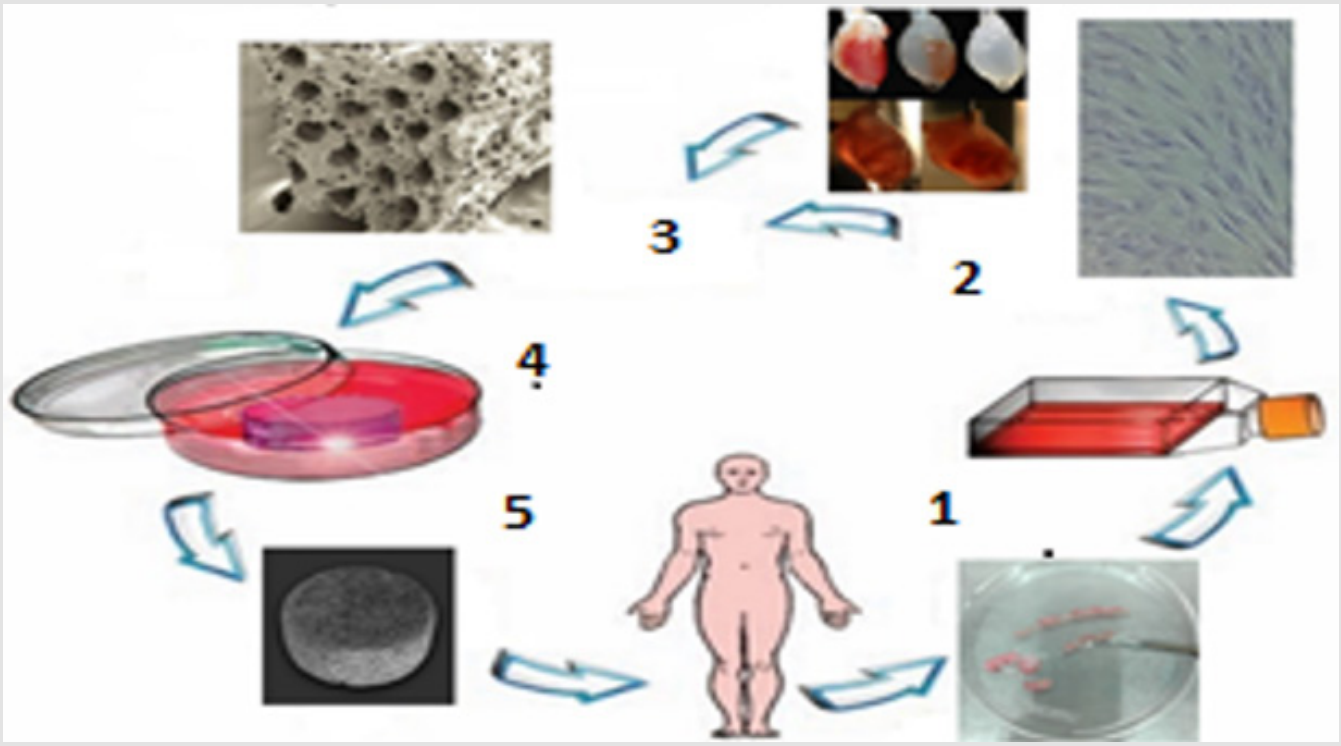

Figure 1: Scheme of general procedures of genetic engineering: 1: cells isolated from the tissue; 2: expansion of the cells in culture, 3: stimulation of the cells with different growth factors; 4: culture of the stimulated cells in vitro for tissue formation; 5: implantation of the cells with the scaffold in the defective tissue structure.

Overview on Stem Cells

Generally speaking there are two main types of stem cells embryonic and adult, where embryonic stem cells (ESCs) can be obtained of blastocysts of the developing embryo and characterized by the potential to differentiate to any of the three embryonic germ layers, which is the pluripotency property, in addition to capacity to be maintained undifferentiated indefinitely [10], ESCs express stem-ness antigens: octamer binding protein (Oct-4), stage-specific embryonic antigen (SSEA) 3 and 4, SOX2, Nanog, LIN28, alkaline phosphatase, rex-1, and crypto/TDGF1, and they show high levels of telomerase activity [11,12], where Oct-3/4, SOX2 and Nanog are crucial for self-renewal [13], where Klf4 and c-Myc genes maintain the pluripotent abilities [14]. Because of this self renewal and plasticity potentials ESCs are good cell lines for tissue engineering and regenerative medicine, and treatment of many genetic diseases as blood disorders [15], genetic immune system diseases, cancers [16], type 1 diabetes [17], Parkinson's disease [18], spinal cord injuries [19]. On the other hand, there are many limitations of using ESC as a source of tissue engineering; firstly would be ethical limitations because of the need to kill the embryo in the process of cells extraction, secondarily the embryo is allogenic to the recipient and immune response would be expected, finally the high probability of tumor generation due to the teratogenic potentials of ESC [20-22].

On the contrary, adult stem cells (ASC) or stromal cells are characterized of being multi-potent, which means they have the capability to differentiate to multiple cell lines but restricted to the same embryonic germ origin, which means they contain a limited proliferation potential. The advantage of the ASC is that they can be autogenic i.e. extracted from the donor then expanded *in vitro* to be re-implanted again, causing no risk of immune reaction, a limitation would be if the tissue to regenerate is already genetically defective like in genetic syndromes, or with limited stem-ness potentials like nervous tissue. ASC have been first isolated from bone marrow and named mesenchymal stem cells (MSCs), but later it was found to exist in nearly all tissues of the human body.

MSCs can differentiate to all cell lines of mesenchymal origin i.e. can be osteogenic, chondrogenic and adipogenic and this property is a main feature characterizing a cell line as a MSCs. MSCs are also hypo-immunogenic and even immunosuppressive they have the ability to suppress the immune system [23,24]. Recently, scientists were able to reprogram differentiated stromal cells to ESC-like state. Takahashi and Yamanaka (2006) using retrovirus could express nuclear transcription markers e.g., Sox2, Klf4, Oct4 and c-Myc in differentiated somatic cells, which gave this cells properties similar to ESCs, and which became widely known as induced pluripotent stem cells (iPS cells). Besides expressing the stem-ness markers, iPS cells are similar to ESCs, in many aspects, like doubling time, chromatin methylation patterns, even ESCs drawbacks like embryonic body (which is 3 dimensional aggregation of pluripotent cells) formation, teratoma formation, potential for chimera formation (which is two genetically distinct structure in the same organism [25].

ESCs like stromal cells will allow us to overcome the limitations hindering the use of ESCs in regeneration therapy which would favor the use of iPS over the ASC in tissue engineering, they are useful tools in drug development, diseases modeling, tissue regeneration and even transplantation medicine. But a drawback of this method is the concerns of using viruses to induce the genome of stromal cells to become ESCs like which can lead to tumor genesis, suggestions been made to control this drawbacks by directly delivering proteins or transient (removable) vectors [26-31]. Another drawback with iPS is the recent reports of its immunogenicity *in vivo* although its autogenicity [32], which means that a long way of research and studies are essential before any thoughts of using these cells in clinical trials Figure 2. The Mesenchymal Stem Cells (MSCs) have great potential for use in tissue engineering and regeneration and became very attractive alternative treatment modality in the treatment of periodontal defects. MSCs are adult stromal cells that have multi-potent abilities, under certain conditions can differentiate into different cell lines. There are many definitions given to stem cells, ranging from a very simple as unspecialized cell that gives rise to differentiated cells, others more specific definitions: stem cell is a "clonogenic", relatively undifferentiated cell, which have the ability to self-renew and multi-lineage differentiation [33].

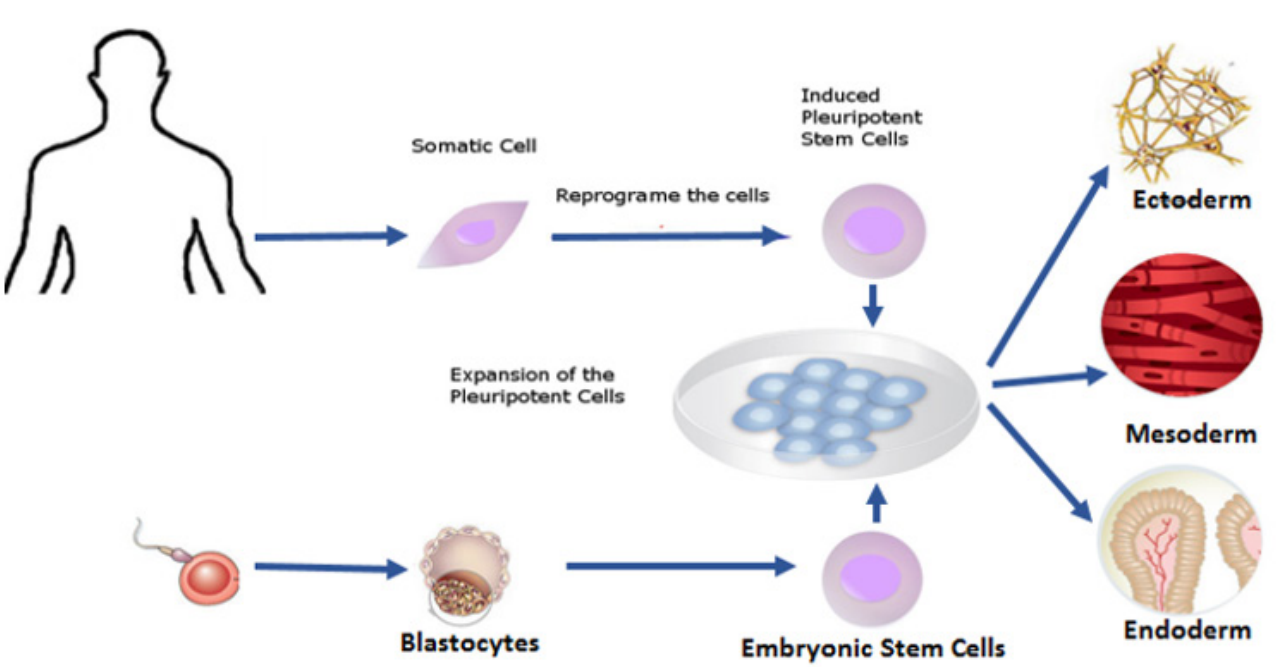

Figure 2: Show comparison flow chart of using Embryonic stem cells and induced pleuripotent stem cells in the differentiation of the 3 germ cell lines.

By definition, stem cell can self-propagate and give other stem cells which is its clonogenicity, meanwhile its progeny can maturate and differentiate to different cell lines that can give rise to a wide range of specialized cells. MSCs express several sole markers like: CD73, CD90, CD44, and CD105, and they lack hematopoietic cell markers like CD34 and CD45. MSCs low express major histocompatibility complex (MHC I), while totally negative for MHC II [34]. It has been shown in many studies that injection of *in vitro* expanded MSC home preferably to sites of tissue damage, supporting healing and regeneration, the chemotactic signal for this event is still not fully understood [35]. These make MSCs ideal cell source for tissue engineering [36]. The approaches used to identify MSCs were the use of selected markers expressed by the cells *in vitro* to find out positive cells *in vivo*, which although sensitive is nonspecific as most of the cell markers are only specific in certain conditions [37,38] or the infusion of already marked cells in culture

in an animal model and study its distribution in the tissue, which though less accurate to precisely study the MSCs distribution in the body and this is because the cells may get attached nonspecifically in different positions *in vivo* [39].

A third approach would be isolating MSCs systemically from organs and tissues, evaluating its characteristics, but this approach cannot allow visualizing the MSCs distribution in the organism consistently as there is no one study isolated the stem cells from the body as a whole but in the form of isolated studies in different organs and tissues. Reservoirs of stem cells have been found in all tissues of the body postnatal contributing for maintaining the tissue of the body, allow for tissue injury regeneration, in the epithelium stem cells were found in the epidermis and in intestine [40]. In the nervous system, stem cells have been found in the central nervous system [41], even in the muscles satellite cells have been detected [42] and in the bone adult bone marrow, which was the first tissue to isolate the stem cells from [43] contain two types of stem cells, which are hematopoietic stem cells [44] and MSC [45]. Adipose tissue is easily accessible and most abundant source of stem cells, and with very rapid *in vitro* stem cells expansion [46,47], tendon [48], periodontal ligament [49], lungs [50], synovial membrane [51] and gingival tissues [52]. The nature and localization of MSC in different organs of the body is not fully understood yet, even the exact origin of them is still debatable; however, there is a growing evidence of very close relation of MSC to pericytes [53,54].

The MSCs are widely distributed than it was previously thought. Evidence-based results show that their main position in the tissues is in the vessel walls and around the blood vessels, excluding a possibility that MSCs were partially or totally from the circulating blood were ensured by performing intravascular perfusion for the animal models before organ resection, and that no long term culture have been established from blood collected of the animals as the study control [55]. In summary stem cells are now considered a corner stone in the science of tissue engineering, and this is attributed to their high differentiation to different cell lines capability, beside their ability to clone themselves rapidly and migrate throughout the scaffold. Mainly there are two types of stem cells, namely Embryonic stem cells, which are active in cloning and differentiate to the three germ lines with embryos, but many ethical and medical factors limit their use in tissue engineering, other source of stem cells would be adult stem cells which were first discovered in bone, and now was found to exist in nearly all the tissues of the body, their multiplication and differentiation capacity differ according to their tissue of origin and age of the subject, adding to the fact that they multipotent not pleuripotent, which means they are dedicated to differentiate only to different cell types of the germ tissue of origin. Recently embryonic stem like cells were created, by reverting stromal cells to embryonic status using genetic engineering which would be a very promising source to tissue regeneration but still needs more development, as nuclear programing of cells by viral transfection elicits many risks of tumorogenicity and immunogenicity.

Stem Cells and Biomaterials

Biomaterials scaffolds, which stem cells grow through, allow us to regenerate tissues. This is exactly the main objective of tissue engineering. By definition, biomaterials are natural or synthetic materials interacting with the biological systems [56]. Initial biocompatibility studies concentrated on seeking materials with least chemically reactivity. With the development of the biocompatibility research, this goal has changed to seeking biomaterial that can interact with the tissue and degrade over time changing biocompatibility definition to "materials with appropriate host response" instead of limiting the host response [57]. In fact, tissue engineering applications entail that biomaterials meet several criteria like appropriate mechanical strength to withstand forces during functions, wide surface area for stem cells attachment, ability to degrade over time concomitantly with replacement with normal tissue that regenerate, stem cells scaffolds must be able also to be sterilized to control contamination of the regeneration process [58]. Bone regeneration scaffolds must have another property, which is tailorable interconnected porosity to allow stem cells and vascularization to go through the scaffold and grow into desired morphology, typically 90% porosity with minimum diameter of 100 microns are recommended, while in fact net shaped scaffold materials are the cost-effective standardized shape suitable for mass production industry of biomaterial in the market field [59-61], Table 2.

Table 2: Characteristics of ideal scaffold material for stem cells bioengineering.

The characteristics of ideal Stem Cells Scaffold Biomaterial
Minimum chemical reactivity
Induce tissue growth on its surface
Degrade with the same degree with new tissue formation
High mechanical strength to withstand functional forces
Surface irregularities to increase its surface area for better attachment of stem cells
Network of interconnected porosity which allow stem cells and vascularity to penetrate through the scaffold
Scaffold of different pore diameter in different depth of the material, to mimic the architecture of the normal bone

After extensive research on biomaterials, it became crystal clear that synthetic materials can't completely mimic the natural bone, and this is because bone as a tissue is extremely complex structure in every aspect. Adding to that, an important shortage in the synthetic biomaterial is the lack of the ability of self-repair, compared to what the living bone can do [62]. It impossible to have all the properties needed for a biomaterial in a unique material composition, so a possible solution of this issue is by utilizing a synergistic approach using different materials by combining different properties, in a composite material. The ability to mimic the struc-

ture of the natural microenvironment of the extracellular matrix of the connective tissue using nanoscale biomaterials can provide optimal biomimetic topographic structure for stem cells and so make it possible to regenerate tissue with similar properties of the native tissue. We can summarize problems shown with synthetic biomaterials as host response causing long healing period (more than 6 weeks), physical and biochemical properties that is not completely mimicking the natural bone, lack of total vascularization, which result in decrease in bone growth and so osseointegration, susceptibility to infection and septic loosening [62].

Stem cells interact with the biomaterial scaffold in two levels for optimum tissue engineered regeneration process: First, *in vitro* after initial cell seeding in the lab and second *in vivo*, where the expanded cells in the lab with the scaffold interact with the host tissues after implantation. A material cannot be named biomaterial, except after being sure of its biocompatibility, i.e. does not elicit immune reaction or have any cytotoxicity. We can categorize biomaterials into inert (tolerant) materials, which can't stimulate any biological bond between them and natural bone; they are only biocompatible, second would be bioactive materials which can stimulate , in a chemical and/or biological way, natural bone regeneration, i.e. they are not just biocompatible, and lastly resorbable materials, which are characterized by being resorbed and get replaced by natural bone [63] Figure 3. In order to be able to regenerate and/or augment bone, a good understanding of bone healing process, and principles of bone physiology must be attained, because bone remodeling-process depends on the amount of forces exerted on them, known as Wolff's law, which keeps the bone shape and density, that would explain why disuse of bone for long duration causes atrophy, a phenomenon like osteoporosis [64].

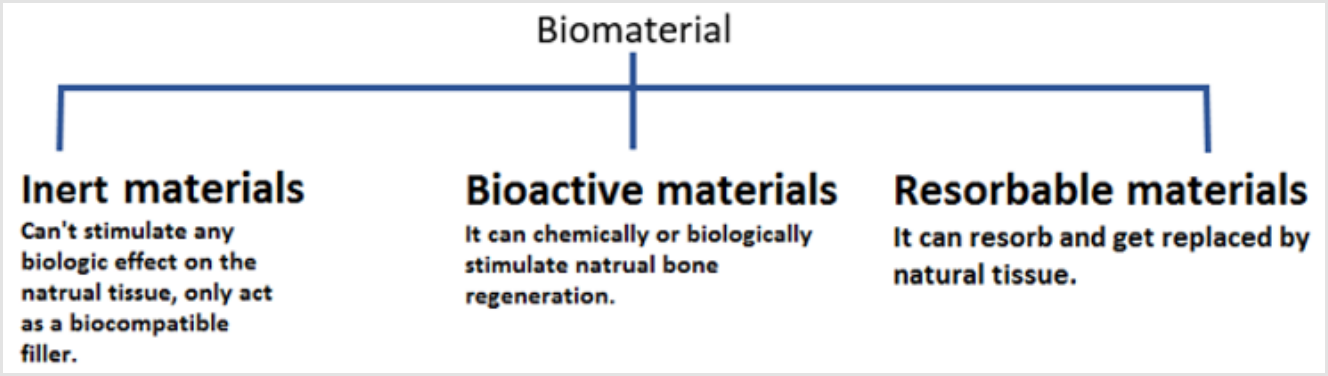

Figure 3: A diagram showing different types of scaffold biomaterials.

Forces exerted on bone during function like compression, tension and flexion by itself and by stimulating interstitial fluid inside the bone create forces and deformation at microscopic level, which stimulate the osteocytes, and which was hypothesized to stimulate the differentiation of stem cells into osteocytes with subsequent bone deposition. In a study where cyclic strain of MSC using equi-biaxial cyclic strains (3%, 0.25 Hz) cultured *in vitro* in osteogenic media caused 2 to 3 fold matrix mineralization when compared to control and decreased proliferation rate, as it were found to stimulate the extracellular signal-regulated kinase (ERK1/2) and p38. This effect is because inhibiting ERK1/2 decreases calcium deposition by 55%, and inhibiting p38, which stimulate osteogenic phenotype, suggesting that p38 might has inhibiting effect in strain-induced osteogenic differentiation. This shows that mechanical stresses induce MSC differentiation, emphasizing an important role of physical stimulation on bone tissue regeneration [65]. Also, it has been shown that mechanical strains inhibit MSC adipogenic differentiation through β-Catenin cell signal stimulation [66].

In summary, we can recognize that scaffold are essential asset for biological engineering, and tissue regeneration. An ideal scaffold biomaterial would fulfill the goal of interacting with the natural tissue, stimulating its growth over its surface, while degrading with same degree, performing this while maintaining favorable mechanical and physical properties that can withstand the functional loads applied over it acting as shield that is protecting the weak regenerating natural tissue. Porosity is an essential property that has to be attained in an ideal scaffold biomaterial, with the aim to allow stem cells, vascularity, fluids and growth factor to go through the depth of the scaffold biomaterial. Lastly studies done on bone healing physiology showed that mechanical strain on bone has a positive effect in osteogenic differentiation of MSC through stimulation of ERK 1/2 and p38 pathways, also it downregulate adipogenic differentiation of MSCs through stimulation of beta catenin cell signaling pathway.

Overview of Tissue Engineering Scaffolds

Scaffold characteristics are governed by its physical nature and its manufacturing process, where different materials have been used as a scaffolds in tissue engineering; metals (e.g. titanium), ceramics (e.g. hydroxyapatite), glass (e.g. bone glass), polymers, either natural or synthetic, and composite scaffold of more than

one material. The scaffold material will govern its physical and chemical properties, such as its mechanical strength [67-71], and its degradation [72-75], while its porosity, which allow drugs and cytokines delivery, will depend on the fabrication process [76-81], these properties and requirements were extensively reviewed as shown above. Scaffold differ in many aspects, regarding its material they are either synthetic or biologic, regarding degradability they can be biodegradable or not, this characters will affect the uses of the scaffolds in different tissue engineering procedures [82]. A big obstacle in scaffolds design is the fact that biodegradable materials are weak, while strong materials most of the time are not biocompatible [61].

Regarding scaffold material porosity, features like interconnectivity, size of pores, and roughness of the surface of the scaffold, have a direct effect on stem cell interaction with the scaffold, the degree of nutrients reaching the cells through the scaffold and cells waste removal [83]. Porous scaffolds must meet some requirements, where they must have adequate stiffness and strength to provide essential primary load bearing, and it should stimulate tissue regeneration, these goals are approached by the scaffold general design, surface modification, ability of local delivery of drugs and/or cytokines, and possibility to reshape it to fit the pathological defects. Regarding the pores themselves they must fulfill some critical issues, first the porosity must be uniform and gradient; second it must not affect the mechanical properties of the material extensively like modulus of elasticity and strength. Pore sizes ideally should be between 40 to 100 microns and well distributed, pore wall with uniform thickness and pores has to be interconnected, which allows cell migration in different direction *in vitro* and *in vivo* [84]. Material requirements that must be fulfilled to meet tissue engineering criteria like biocompatibility to allow stem cells attachment, growth and proliferation, ability to create strong bond with natural bone, the rate and ability to biodegrade must match with the rate of new bone formation, the material must have good mechanical properties adequate for load bearing areas while bone healing is taking place [85], Figure 4.

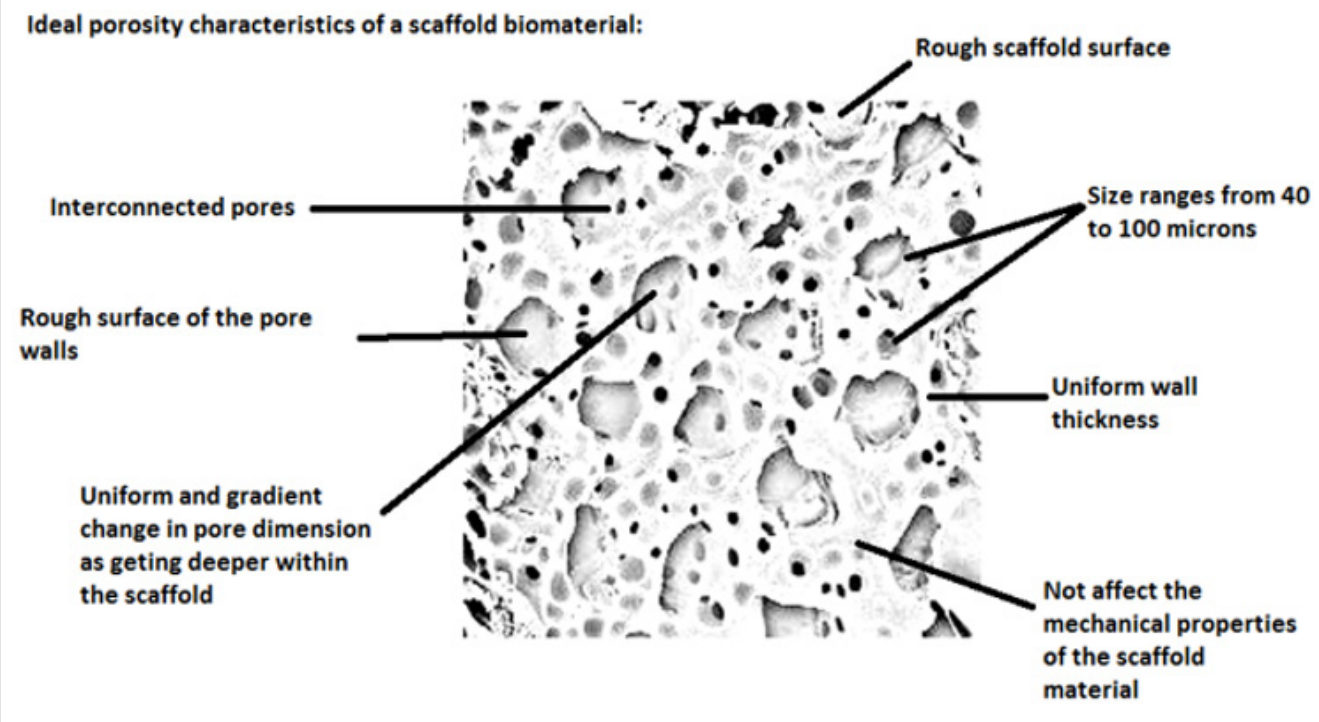

Figure 4: Diagrammatic representation that summarizes the ideal characteristics of porous scaffold biomaterial.

Ceramics as Biomaterials

A major class of biomaterials for bone repair is ceramics, such as hydroxyapatite and tri-calcium phosphate [86,87]. Ceramics have great biocompatibility characteristics; they can exhibit stable or resorbable properties, are not metals so they do not corrode, they have very high hardness, and compressive strength, besides ability to obtain good bioactive properties, and therefore this makes ceramics very highly biocompatible materials. On the other hand, they have many disadvantages, being very stiff, easily fracture due to low toughness, beside being with higher modulus of elasticity higher than bone, which create significant difference in stiffness between them, which disturb the load distribution exerted on natural bone and ceramic grafts, where ceramics get most of the loads instead of bone, making ceramic acting as a shield [88]. This leads to considering HA to be unsuitable for use in load-bearing appliance in orthopedics, instead it been used to coat strong

load-bearing metal implants to increase its biocompatibility or composite scaffold materials, such as polymers.

Polymers on the other hand have good modulus of elasticity, and stiffness, in addition of the great biodegradability, but most of polymers are very weak in bioactivity and so cannot osseointegrate with natural bone readily, so compromising the advantages and disadvantages of ceramics and polymers, by incorporating the two materials, in a composite graft or scaffold, would be a way to overcome the drawbacks of both. Polymer scaffold uses depend on its properties, which depend on its composition and its macromolecules arrangement [88]. In a recent study by Danoux and his co-workers in 2014, aimed to assess the bioactivity of bone scaffold composite polymer of polylactic acid (PLA) and hydroxyapatite (HA), where they did *in vitro* and *in vivo* studies, they mixed 50 weight % PLA with 50 weight % nano HA forming a homogenous composite polymer, they immersed this composite *in vitro* in 2 solution, simulated physiologic saline and simulated body fluids, they found after 12 weeks of immersion that PLA/HA composite showed more loss of weight when compared to control PLA particles, also they observed that composite in physiologic saline solution was continuously releasing calcium and phosphate ions, while in the body fluid solution they recognized on the surface of the composite formation of calcium phosphate layer, with less calcium and phosphate ions in the solution in both the PLA/HA and PLA groups.

They cultured MSCs on both PLA/HA composite and its control PLA particles, aiming to assess the bioactivity of the composite polymer, and they found that both support the proliferation of MSCs after 2 weeks of culturing, but when culturing the cells in osteogenic medium, the cells cultured over PLA/HA composite showed more alkaline phosphatase activity compared to control. They also did *in vivo* study on dog animal model, where they implanted the PLA/HA composite intramuscularly for 12 weeks period, they found that the composite implant was osteoinductive of heterotopic bone formation, which wasn't the case with the PLA control which showed no osteoinductive capacity, also they noticed that PLA degraded more profoundly *in vivo* in comparison to the PLA/HA composite, which wasn't the case *in vitro* [88a]. Boutin studied alumina and zirconia as biocompatible ceramics in 1972, which showed great biocompatibility, durability and mechanical strength, but on the other hand loosening of the implants in the natural bone took place later on, this of course caused many clinical failures, and the explanation of this finding was that ceramics are inert, and were implanted without bone cementation [89].

Trying to overcome these drawbacks, the biologically active biomaterials were then developed, as calcium phosphate ceramics [90], bioglasses [91] and hydroxyapatite (HA). Being similar to the mineral component of natural bone, they showed good osteoconductivity and bone bonding ability [92]. However, the main limitation for the use of hydroxyapatite ceramics was their inherent brittleness and difficulty for processing [92,93]. HA is considered to be the most important bioceramic due to its high bioactivity and stability, not like other calcium phosphates, being with high strength, it does not break down under normal physiological forces. Hydroxyapatite is a mineral with a chemical formula of $Ca_5(PO_4)_3(OH)$, but its usually written as $Ca_{10}(PO_4)_6(OH)_2$ to show that its crystal is constructed of two parts, it is created of calcium apatite. The bone mineral, which can be considered as a basic calcium phosphate apatite represents 50% of the bone dry weight, where the bone HA is not identical to that found in rocks in nature, where it is different by 50% in volume and 7% in weight and called bone mineral [94,95], while the enamel and dentin HA is calcium deficient carbonated hydroxyapatite. HA is extensively studied as inorganic material and is famous of being bioactive, highly osteoconductive, with high strength and modulus of elasticity, and high biocompatibility [96,97].

HA is bioactive which means using it as a graft or implant with natural bone will allow bone ingrowth inside the graft and/or osseointegrate with the natural bone. HA is not stable compound at high temperatures, where it decomposes at 800 to 1200 °C, and this wide range is due to the difference in the stoichiometry of the HA. But, on the other hand, it is thermodynamically stable at normal body temperatures and physiologic pH, which allow it to actively bind to bone with strong chemical bonds. This property gave it the privilege to allow rapid bone repair. We can use the bioactive properties of HA in many biological applications, coating the surface of orthopedic or dental implants, which are usually made of titanium, titanium alloys or even stainless steel, where the body immune system won't generate immune reaction against HA considering it like the bone minerals, the acceptable technique for this application is by plasma spraying, and which is now widely used for commercial applications [98]. The other major use of HA is as bone graft, in the form of granules, or porous blocks, to fill bone defects acting as bone filler, which is used to regenerate lost bone due to disease, or to augment existing bone in reconstructive surgery. In addition, HA porous bone blocks can be used as scaffold to introduce stem cells in tissue engineering therapies, as an alternative to autogenous bone grafts, with minimal morbidity and reduced healing periods.

By its chemical nature HA allow substitution of its structure, so it's common for non-stoichiometric HA to be seen, where the most common that the hydroxyl group be replaced with fluoride to make fluoroapatite, or chloride to form chlorapatite, Fluoride ion exchange easily the hydroxyl group in HA because of two reasons, first fluoride ion is smaller than hydroxyl group, second more affinity of fluoride ions to HA crystal than hydroxyl ions. while this property can also lend a defect to exist in the HA crystals leading to defective hydroxyapatite, but also it has a great advantage of the ability to change in the chemical composition of the material and hence its bioactive characteristic, this character could allow us to incorporate fluoride which shown to have beneficial effect on the stem cell biology, Liu, et al. [92a] did a studied if alignment of apatite

crystal to form enamel like substrate would affect dental pulp stem cells (DPSC) adhesion and proliferation after culturing for 7 weeks on 2 surfaces namely fluorapatite ordered (FA) and disordered surface (nFA), then they did SEM analysis, PCR array and pathway focuses matrix array, after 3 days in culture cells bount over the FA surface showed significantly more number than on the nFA surface, they also notice changes of 20 pathways genes in cells cultured over the FA surface compared to control, the most interesting of them were the genes of cel matrix adhesion molecules, vitronectin receptor integrin alpha V and adhesion protein fibronectin 1 were shown to be up-regulated, which was proved by the SEM study which showed good biocompatibility of stem cells over both surface with only difference that cells in FA surfaces showed cell matrix interaction with the surface [92a].

Another study done by Kushwaha et al in 2012, aimed to study the *in vitro* effect of culturing MSCs on a glass ceramic based on nobium doped fluorapatite, Where they melted the glass 2 times at 1525 °C for 3 h, then casted to form disks, then heat treated to increase the crystallization of fluroapatite. which is then divided into 3 groups, either left with no change or mechanical ground or chemically etched. Cells proliferation and staining for the expression of alkaline phosphatase was checked at 1, 4, 8 days time points, where cells cultured on polystyrene dishes were uses as the control. Mineralization were confirmed by Alizarin red staining and SEM assay, where SEM analysis were done also to assess cells morphology at 2 and 4 days time points. The study showed that initial cell growth on the discs compared to control dishes showed no significant difference, area covered by cells on the discs didn't show significant difference compared to control group when measured at day 8. SEM analysis showed that MSCs showed many filopodia attaching with the ceramic surface or with eachother and showed calcium mineralized granules accompanied with fibrils over the disc at 35 day time point, the authors conclude that MSCs attached, proliferated, osteodifferentiated over fluoroapatite glass ceramic glass without any difference than polystyrene plastic control [92b].

A more recent study on DPSCs on fluoroapatite 3 dimensional culture, namely electrospun polycaprolactone (PCL), nano-extracellular matrix nanofibers with FA crystals compared to controls without FA. When they fabricated the scaffold they emphasized that FA crystals be evenly distributed insdie the scaffolds. After culturing the cells on the scaffolds for 28 days SEM analysis showed initial attachment of the cells on the scaffold with more multicellular aggregates in the FA scaffolds, also after 14 days of culture cells culture on the FA scaffolds showed slower proliferation compared to the control. They noticed that without any mineralization induction media cells cultured on the FA scaffolds showed upregulation of pro osteogenic molecules starting from day 7 namely: dmp1, dspp, runx2, ocn, spp1, col 1a1, with significant increase in alkaline phosphatase enzyme activity at time points 14 and 21 days compared to the control scaffold, while osteocalcin expression was seen only in the cells seeded on the FA scaffolds at day 21, and significantly increased at day 28, which was proved by staining with Alizarin red and Von Kossa. They concluded that FA incorporation in 3 dimensional PCL nanofiber scaffolds showed a favorable extracellular matrix environment that potentiated stem cells proliferation, differentiation and mineralization [92c].

Natural bone is composed of inorganic compound (mainly partially carbonated HA on the nanometer scale) and organic compound (mainly collagen). The nanometer size of the inorganic component (mainly bone-like apatite) in natural bone is considered to be important for the mechanical properties of the bone [99]. Researchs in this field suggested that better osteoconductivity would be achieved if synthetic HA could more resemble bone minerals in composition, size and morphology [100,101]. In addition, nano-sized HA may have other special properties due to its small size and huge specific surface area. Webster, et al. [102,103] have shown significant increase in protein absorption and osteoblast adhesion on the nanosized ceramic materials compared to traditional micronsized ceramic materials. Most mammalian cells are anchorage-dependent cells and they need a biocompatible substrate for attachment, migration and differentiation to form new tissues. Recent research demonstrated that cell adhesion and survival could be modulated by protein pre-absorption on the substrate [102-104]. Therefore, protein absorption is of importance in evaluating a biomaterial for bone grafting, Table 3.

Table 3: Summarizes the advantages and disadvantages of HA as a biomaterial..

Advantages of HA as a Biomaterial
Most close material chemically and physically to natural bone
Can be chemically modified by incorporating fluoride ions in its crystal which be shown to favor stem cells proliferation, attachment and differentiation
Good mechanical properties which would protect proliferating cells
Bind chemically with the natural bone
Can be fabricated with ability to control its physical and mechanical properties
Can be fabricated with different pore sizes and shapes
Allow growth of natural bone on its surface
Can be fabricated with different sizes ranging from nano scale to block sections

Can be heat treated, physically ground or chemically etched to change its surface characteristics after its fabrication
Not immunogenic to the body immunity
Disadvantages of HA as Biomaterial
It's not a biodegradable material
Although it's chemically very similar to bone HA but still it's different than it
Lack the organic matrix of the natural bone
Its mechanical properties is different than natural bone, it's harder, stiffer, torsionally weak, less resilient, high modulus of elasticity, and more brittle than bone
When infected it get occupied more extensively by bacteria creating more severe infection and foreign body reaction of body compared to natural bone

Hydroxyapatite Fabrication Methods

There are different methods for manufacturing nanoHA, generally there are 2 main pathways: chemistry and high thermal processing. The wet chemistry procedure depend on precipitation of calcium and phosphate by suspending the objects wanted to be covered in supersaturated solution of calcium and phosphorous, the advantages of this method is that its easy, cheap and doesn't require special equipment, while its main disadvantages is time consuming , unreliably in control the nature of particles morphology and crystallinity, and the size vary significantly but its said to be usually less than 1 um [104b-104d]. The sol gel technique is a method where a solution containing colloidal particle which by decreasing its surface charge it become an amorphous gel which is solidified by drying then heated to 600 C to get rid of remaining organic material [104e], it is the technique used to synthesis the fluoro-HA by reacting Calcium phosphate with Calcium Fluoride at 900 C, which is preferred due to its improved mechanical properties and high thermal stability than HA, plus stimulating MSC proliferation and osteogenic differentiation [104f]. The hydrothermal method HA is synthesized by converting slurries to crystalline phase under high pressure and temperature, to form particle size of about 1 microns [104g]. The metathesis method is a combination of the chemical and physical pathways, where chemically precipitated HA on the substrate in aqueous solution, is then exposed to electrophoresis where the charged particles migrate to counter charged electrode which create HA coating with particle size less than 50nm [104h,104i].

Lastly plasma spraying method of HA synthesis where argon is coverted to gas plasma using direct current arc, this creat enormous heat of the powder which is then propelled over the substrate, although its wide commercially used due to its economic efficiency and reproducibility, it has low mechanical properties [104j], and this attributed to the fact that an amorphous layer is created on the top of the coating due to cooling which is significantly different than the underlying crystalline stricter of the coating [104k], adding to that the enormous heating that caused by plasma lead to dissociation of HA into many Calcium Phosphate phases which affect its solubility in the physiologic fluids [104k]. Regardless of the fabrication method of HA there are many procedures which are done to have tailored characteristics of it, sintering is done by exposing the material to high temperature which causes increase of its hardness, reduce cystal size and shrinkage, eliminate acidic phases, water, and organic materials. Sintering drawbacks would creation of multiple phases of the material and cracking of the HA coat on the substrate [104l]. Another procedure that been used recently is ultrasound irradiation to microsized HA clumps to form ultrafine HA slurries, in 2009 Poinern, et al. [104 m] found that when ultrasound irradiation is used with chemical formation methods a control of particles size and morphology could be achieved, where 30nm sized particle can be successfully produced when using 50 W ultrasound irradiation power at 400 C temperature [104m], Table 4.

Table 4.

Method	Particle Size	Main Features
Wet chemical	0.07-0.64um	Simple, cheap, slow and can't control particle size or morphology
Sol gel	Nano-size	Can't get rid of impurities
Hydrothermal	1um	Can't control particle size or mophology
Metathesis	50nm	A combination of chemical method with electrophoresis
Plasma spraying	200-300um	Create uniform coating, but with multi-phases

Hydroxyapatite at Nanoscale

Bone is designed architectually to be composed of inorganic collagen matrix which surround a nanoscale hydroxyapatite crystals ranging of 20 to 40nm, which gives the bone its unique characteristic of being strong but still with some degree resilient. This nano HA component of bone allow bone remodelling by osteocytes signalling, by adsorption of proteins of the estracellular matrix that stimulate the adhesion of osteoblast with subsequent proliferation and differentiation [104n]. That's why it sounds

normal that in regenerative therapy, body would act more favorably dealing with HA of same size of normal bone HA. Many studies have found a positive effect of nano sized HA, in study done by Sun et al of 500 to 300 nm size nano HA they found that it had a negative effect on cells activity, namely inhibitory effect on osteoblasts cultured in-vitro over this particles [104o], Cai, et al. [104p] did a study of *in vitro* culturing MSC of bone marrow origin over nano HA particles of size 20, 40, and 80 nm where they found it had a stimulatory effect on cells vitality and proliferation [104p], not only the nano sized HA particles which had this effect, but even nano sized surface roughness topography were found Balasundaram and this co-workers to have stimulatory effect of osteoblasts proliferation and differentiation [104q].

Webster and his group had a series of studies on the biological effect of nano roughness size less than 100nm, in there first study they found that nano rough HA prarticles when compared to non rough particles of the same profile i.e chemistry, phase and crystallinity, they found that the nano rough HA have positive effect on cell response and hence increased osteointegration, also in this study they found that of all serum proteins vitronectin which is an essential protein for osteoblast adhesion, adsorp to nano phase alumina, while serum albumin adsorb to the conventional size alumina [104r]. In another study they did with same surface roughness size, they found it stimulated osteoblast adhesion, proliferation, and increase of differentiation marker like alkaline phosphatase, compared to standard HA particles with surface roughness more than 100nm [104s], and lastly in study to experiment the effect of nanosurface roughness they found that a phenotypic marker of osteoclasts called tartrate-resistant acid phosphatase synthesis was upregulated and resorption pitting increased in the cells exposed to nano surface roughness of less than 100 nm compared standard HA particles [104t].In a mini review done by Fox et al in 2012, they summarized the applications of nano-HA into 4 main fields, namely: Hard tissue repair, drug carrier, antibacterial, magnetic composites, gene therapy [104u]. One of the major limitations of using HA as a nanoparticle in bone grafts is the risk of atherosclerosis induction. Coronary artery calcification is a common feature of atherosclerosis and is due to the intimal deposition of basic calcium phosphate crystals, consisting mainly of calcium HA [105-109].

Intimal calcification has tended to be seen merely as a surrogate marker for the burden of disease rather than as a driver of atherosclerosis. However, recently been demonstrated that basic calcium phosphate crystals (consisting of pure calcium HA) stimulate release of TNF and other proinflammatory cytokines from human *in vitro*-differentiated macrophages [110]. Since TNF, in turn, promotes calcification of smooth muscle cells [111], crystal-activation of macrophages may contribute to a vicious cycle of inflammation and calcification in the vessel wall. A hint that smaller calcific deposits may play a significant role in plaque rupture has come from the work of Kolodgie and Virmani. This group has noted that heavily calcified lesions only rarely trigger thrombosis and that the majority of ruptured coronary plaques (>65%) contain "speckled" calcific deposits [112]. It is therefore possible that coronary plaques with only modest calcification are more prone to inflammation and rupture [113,114]. Consistent with this idea, histologic examination of coronary endarterectomy specimens has shown that foam cells are closely associated with small "stippled" calcifications even in early type II lesions [115]. Furthermore, whereas all ruptured coronary plaques contain detectable calcific deposits, the majority of such deposits are "speckled" (>65%), with only a minority of ruptured plaques presenting with more diffuse calcification. Collectively, these observations suggest that moderately calcified coronary plaques containing microscopic HA deposits are more prone to inflammatory activation of macrophages [116,113]. This is also consistent with the idea that diffusely calcified plaques may be more stable, due to resistance to mechanical shear [117].

Porous Hydroxyapatite as Scaffold

Leon defined porosity as the void or space percentage in a solid [118], porosity is a morphological feature of the scaffold not related to its material but more related to the fabrication process. Porous HA, versus dense HA, has much more medical uses like tissue engineering and drug delivery, as porous HA is more similar to natural bone. Also porosity increases the bioactivity by facilitating bonding by providing mechanical interlock between the graft and the bone, which enhance the success rate and the strength of the graft by fixing the graft material firmly with the natural bone. In addition, pores allow regenerated bone to grow through the pores, which also increases the graft strength and pores encourage stem cell attachment, and natural bone osteocytes to penetrate and grow within the scaffold. But porosity in essence decreases the overall density of the HA, which decreased its mechanical strength, this makes its use as an implant material not possible, because is not a good candidate material as a bone graft [88]. However, the long-term biocompatibility *in vitro* and *in vivo* of highly porous scaffolds is not totally understood, especially regarding its degradation and ion leaches of the inorganic phases kinetics.

Porous HA is more osteoconductive and resorbable than dense HA. Synthetic HA bone replacement scaffolds and grafts are porous, simulate the natural bone structure, besides it has larger surface area, which enhances stem cell attachment and hence allows bone growth and regeneration. It has been recognized that different pores size and distribution in HA scaffold would have different tissue engineering applications, where very small pores, with sizes less than 1 micron, would be suitable for protein insertion in the scaffold and hence increases its bioactivity. Moderate size pores, between 1 to 20 microns, would enhance cell attachment, which will direct the cellular growth and large size pores, between 100 to 1000 microns, would allow cells and bone growth. When pore sizes exceed 100 microns bone penetrate through it, which maintains

bone's vascularity and viability [119]. In a classical study, Hulbert, et al. [120] studied calcium aluminate pellets with porosity of 46% in canine bone, where they concluded that pore size of 100 microns is considered the minimum size needed to regenerate bone in general, and pore sizes between 100 and 200 microns appeared with considerable bone ingrowth in the pores. Smaller pores of size 75 to 100 microns, showed ingrowth of osteoid unmineralized bone, while very small pores ranging from 10 to 75 microns did not demonstrate any bone ingrowth but showed penetration of fibrous tissue [120].

Alhough this can be explained due to the effect that the pore size in the normal haversian canals is about 100 to 200 microns, but pores size of 100 microns are not critical size of pores in the scaffolds, as these results are not replicated with other materials in other experiments. A porous scaffold with ideal properties would be with resorbable superficial surface with pores less than 1 micron, followed by a layer with pores of 1 to 20 microns, and at the core the pores should be of 100 microns and more, while the fabrication of a scaffold with size gradient pores sizes would be challenging but using the modern computerized technologies, this could be feasible (Table 5). For new bone formation the surrounding tissues penetration and easier vascularization are essential for the deep cells penetrating the scaffold to survive *in vivo*, which entail adequate macroporosity must be developed in the scaffold, with connection inbetween, which is even shown to be more important than the pore size for the osteoconduction property of the scaffold [121]. On the other hand, if pore size is very small it get blocked by the cells preventing nutrient delivery and waste removal to the deeper cells and so prevent new tissue formation [122]. Porosity is also essential for the integration of the scaffold into the natural bone as prevent the movement of the implant till the new bone is formed at the interface, this factor acts as a biological fixation method of the scaffold to the natural bone, which is highly dependent on the scaffold porosity. *In vivo* experiment in a rat model using solid versus porous HA particles as delivery vector for bone morphogenic protein 2 (BMP-2), no new bone was found on solid particles in contrast osteogenesis was found in the porous particles [123,124].

Table 5: Different pore sizes and its effect on hydroxyappatite osteoconduction properties.

Pore size (in Microns)	Particle Size	Main Features
Less than 1 micron	Microporosity	Intial protein adsorption
1 to 20 microns	Microporosity	Aid in cells attachment and direct cell growth in proliferation and growth stage
100 to 1000 microns	Macroporosity	Aid in connective tissue and bone in growth

Coating titanium alloy implant with porous HA did not show increase of osseointegration in the canine mandible, but caused osseous interlocking with HA micropores, while in the maxilla, there were more bone, which may indicate that HA micropores may have better effect in poor bone quality. It is clear that large pore sizes (450 microns) increase bone deposition, pore wall roughness and micropores (10 microns) are also very important, as HA rods with pores size of 200 microns but with smooth dense walls could not stimulate ectopic bone deposition in dogs in comparison to rods with 400 microns rods but with rough pores walls [125]. Microporosity besides increasing the surface area induces higher protein absorption and ion exchange, which deposits apatite by dissolution and re-precipitation [61]. In a study that was comparing same pore size with random pore scaffolds of same material *in vivo* in cranial defects in rabbits there were no statistical difference in bone formation, but the architecture of bone was different between the two groups. Specifically, a continuous bone formation was found in random pore size scaffolds, while same sized pores and solid wall groups showed discontinuous bone formation in the form of islands through the scaffolds, which is thought to fasten the healing of the bone throughout the defect [126]. Polymer replication method for fabrication of Biphasic Calcium Phosphate (BCP) with 80% HA and 20% beta TCP with 70% interconnected porosity having a combination of macroporosity 68% of 400um diameter and microporosity 3% of 0.7um diameter found to be supporting new bone formation in immune deficient mice [126a]. Another study showed that a combination of macro-porosity of size ranges of 250-350um, and micro-porosity of size 2-8um resulted in the formation of lamellar and woven bone a property which is abscent in scaffolds without micro-porosity [126b].

Composite Scaffolds

Its scaffolds which are made of 2 or more different materials like ceramic and polymer, achieving the advantage of good mechanical properties of ceramic and the degradation, biologics advantages of polymers, increasing the scaffold toughness, compressive strength, mechanical integrity, and bioactivity such as to be similar to natural bone. In a study on HA and poly-ester urethane (PU) composite scaffolds, it was shown that its being able to adsorb bovine serum albumin (BSA), fetal calf serum (FCS), bovine fibrinogen in considerable amounts in-vitro, they found that the sustained release of BSA could be detected even after 2 weeks and which was attributed to PCL boating, when compared to control PU scaffolds [C1 55]. Another study done using micro CT (uCT) showed that 200um pores of HA/PU scaffolds can be fabricated with 90% volume, by using the salt leaching process, also they found that HA/PU scaffolds showed blood vessels growth of 49cm cm^-2 in the borders and 0.4cm cm^-2 in the central zone compared to 55 and 3 cm cm^-2 for the PU after 14 days of implantation in mice dorsal skin, and which didn't show statistical

significant difference in angiogenesis [C2 56]. In a study where surface modification was done using BCP porous scaffold HA/PCL, it was shown to increase the compressive strength twice, also it encouraged the differentiation of bone MSCs with upregulation of osteogenic gene expression namely osteocalcin (OC), Runx2, Col I, bone sialoprotein, and alkaline phosphatase activity, they were also found to be suitable for protein delivery [C3 54] [126c].

Porosity Fabrication Techniques

The stem cells scaffold must be characterized of offering optimum environment for cells growth, both in-vitro where cell expansion take place, and *in-vivo* after being implanted in the recipient body, and this fact is essential as the concentration and gradient of nutrients, fluid velocity, and transport mechanism is different, also it has to be emphasized that *in-vivo* diffusion is the main mechanism of nutrient and waste products transportation, while in-vitro fluid flow is the principle mechanism. An important criteria that govern the success of a scaffold would be its ability to allow high nutrient concentration within its core. Another important criteria is to have a uniform interconnected meshwork of pores, as a non-uniform porous scaffold creates regions with insufficient nutrient concertation which hinder cells proliferation and tissue growth [88]. There are many techniques implemented to create porosity in a biomaterial, like freezing, drying, sintering, salt leaching, gas foam, and phase separation, thermal induced phase separation, microsphere sintering and scaffold coating [126d, 126e] Figure 5.

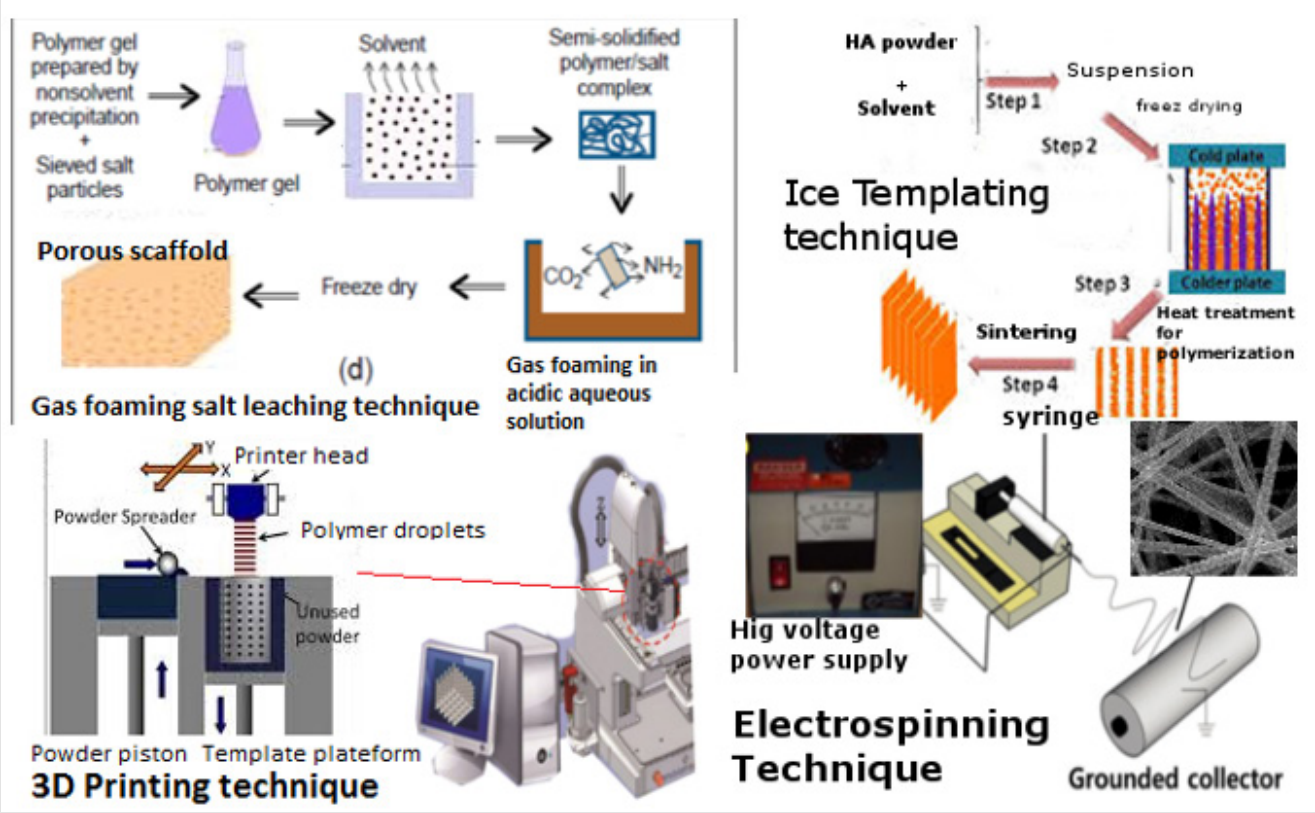

Figure 5.

Each fabrication process depends on the physical and chemical structure of the scaffold material itself. To improve the mechanical properties of bioceramics with fabrication method have been tried in many techniques with no great success to have porosity without affecting the scaffold mechanical properties especially compression strength and modulus of elasticity to approach those of the cancellous bone. The fabrication techniques can be categorized into two main categories, chemical-based approach, and the engineering-based approach, while the first one creates uncontrolled heterogeneous pores, and the second category can produce tailored pores.

3D Printing

Computer aided design (CAD) data systems which are usually named rapid prototyping which fabricated physical objects uniquely by adding materials in layers, with the advantage of creating complex geometry with no need of assembly, and with relatively fast process. In this procedure first a CAD design is done, then powder is delivered to the fabrication piston bed 1 layer at a time, which creates loose powder layer, then is transferred to an inkjet head dispenses polymeric or liquid binder to specific areas leaving the rest of the powder loose, the fabrication piston then

moves down, and the process repeated creating another layer. Then the binder is cured using high temperature and the loose powder is removed by agitation. Finally sintering is done binding the particles together, evaporating the binder to create a 3D structure identical to the computer design [126f,126g].

Electrospinning

Its considered the most effective method for fabrication of micro and nanofiber, and polymeric fibers on a large scale, where polymer is injected by a needle at a critical voltage difference, creating an electrically charged jet, which is spattered on a grounded rotating target which collect the polymer fibers, this creates fibrous scaffold with high surface area and porosity, that mimic to a great extent the natural extra cellular matrix [126h,126i]. Although this technique was initially designed for polymer scaffold, it was successfully used to fabricate ceramic polymer composite [126j-126l].

Ice Templating

In this method colloidal suspension of HA particles are molded in a non porous mold, which is rapidly frozen, then sublimated to get rid of the frozen solvent under very low temperatures in vacuum (Figure 6). In Deville et al meta-analysis done in 2015 with the topic of the mechanical properties of ice templated ceramics and metals, they postulated that ice templating is a versatile and flexible process, that allow a very wide range of porosity size fabrication, with the ability to have a directional pores with constant cross section , or with progressive increase of the pore size as moving along the solidification direction. But on the other hand its considered a complex process where many factors affect its results like solvent nature, powder particle size, PH, viscosity, material nature, surfactant, freezing temperature, and cooling rate. Pores morphology depend on the solvent nature where for example water as a solvent creates lamellar morphology i.e tubular ellipsoidal cross section [124m].

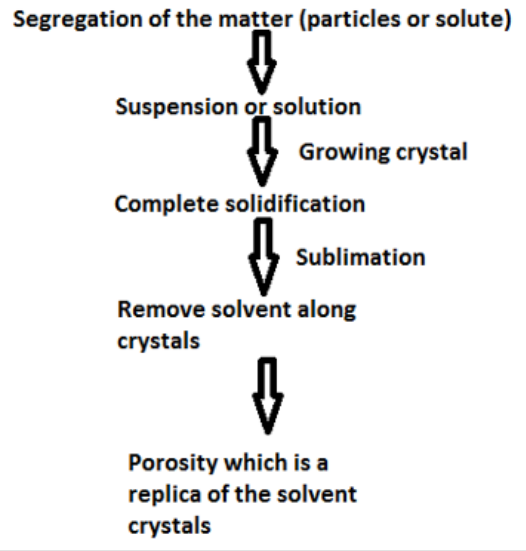

Figure 6: A flowchart that representing the main steps followed during the procedure of ice templating.

Gas Foaming

It is considered a different technique of ice templating where gas is used as porogen to develop the porous scaffold.

Gel Casting

Is a wet ceramic foaming technique where monomer is polymerized in solvent presence forming ceramic loaded body which is then machined in a complex mold as the gel cast is soft to be machined by carbon steel tools [P6]. This green cast is then demolded and dried under controlled conditions [P7], the main advantage of this procedure is the ability to fabricated high quality complex shaped ceramics at a low cost.

Slip Casting

A suspension of fine powder of HA in water or alcohol with 2ry materials like binders and surfactants, which is poured in a plaster mold, which draw water to compact the casting at the mold surface, the advantages of this technique that it provides uniformly dense HA casting with superior surface quality at a low cost, and this is attributed to the fact that the cast has high concentration of the raw ceramic with little additives [88].

Replication Technique

A multistep procedure where a pattern of the desired porous scaffold is made of polymer or wax, which is then suspended in a ring, and then plaster is poured around it, then the pattern is burned out creating a negative mold which is filled with desired liquid phase polymer, that is then hardened by either cooling, curing, or precipitation, the mold is then broke down to have the porous scaffold [88].

At a Time

The design of the scaffold and its material are critical in developing pores in it, regarding its suitability and its biocompatibility *in vitro* and *in vivo*. Developing pores with gradient sizes from the surface to the core with high interconnectivity are essential criteria for porous scaffolds to minimize pore occlusion and allow cell attachment and vascularization, connective tissue and bone ingrowth. The most challenging factor in producing porous scaffolds is to maintain the mechanical properties of the material of the scaffold, as although the existence of many fabrication techniques. The today techniques of a scaffold production, required pore size with biodegradable properties and with reliable mechanical properties, is still not yet achieved. Scaffolds must have well-designed external and internal structures with interconnected pores to allow cell migration as well as connective tissue and vascularity. This pores create a matrix, which serve essential biological functions; first is fostering an immobilized habitat for the growing cells, second hindering unwanted tissue from growing in the wound area by creating protective space for the desired tissues. Finally, act as routes, which guide the migration and growth of the desired cells, which can be aided by the properties of the scaffold material, or with the

incorporation of soluble molecules that would leach out like growth factors, hormones, etc.

More studies are needed to have engineered processing techniques to produce scaffolds with tailored porosity and good mechanical properties and to have more understanding of the relation between scaffold material and its processing and its clinical performance. A better understanding of the processing techniques would affect the biological properties of a material, like for example making osteoconductive materials to be more osteoinductive. Hydroxyapatite as a biomaterial has many characters that suggest it to be a very good candidate to stem cells scaffolding. Its chemical composition which is very similar to the natural bone chemical structure and its mechanical properties which is strong enough to withstand the forces naturally applied on normal human activities, very similar to natural bone, and even can act as a shield protecting the wound during the healing period, its availability and dexterity and ability to be manufactured with different sizes, with solid surface or porous surface, with smooth pores or with rough pore's walls, and even with controlling the pores wall thickness. And finally regarding biocompatibility HA is highly biocompatible, allowing natural bone to grow over its surface, and even with certain pore sizes natural bone would grow inside the scaffold allowing early stabilization of the graft, which can stabilize dental implants. The only drawback of HA as stem cells scaffold would be its low rate of biodegradation lacking important properties that is needed in tissue engineering of simultaneous scaffold biodegradation with new tissue deposition.

Summary

a. The mechanical properties of the scaffold should match that of the natural bone, and which vary greatly between cortical and cancellous bone, where the modulus of elasticity of young bone in cortical plate is between 15-20GPa , while the cancellous between 0.1-2GPa, while the compressive strength for cortical bone is between 100-200 MPa, while the cancellous is between 2-20MPa [S1 1].

b. Interconnected porosity of the scaffold is of utmost importance, to mimic the natural bone, where the cortical bone is 10-30% porous, and the cancellous is 30-90% porous. The minimum diameter of the scaffold pore is 100um [S2 25], however a range of 200-350um was found to be optimum for tissue growth [S3 79].

c. On the other hand porosity reduces the compressive strength of the scaffold and render manufacturing reproducibility difficult, where the material nature of the scaffold would compensate that, where bioceramic materials strength were found to be close to that of cortical bone, while polymers are close to cancellous bone [S4 35]. Generally porosity are assigned to macroporosity which is larger than 100 um, and micro-porosity which is less than 20um [S5 49].

d. Biodegradability is a crucial factor of scaffold success, the scaffold must have the ability to degrade with *in-vivo* at the same rate by which new bone is growing. Multiscale porous scaffolds with macro and micro porosity would be considered an ideal composition regarding biomolecules delivery, biodegradability, and which is considered a major task for the science of tissue engineering [S6 49,50].

e. Incorporation of biomolecules like BMP, FGF, VEGF, TGF beta, in the scaffold facilitate bone regeneration through stimulating the differentiation of MSCs and osteogenesis, plus recruiting osteoprogenitor cells to the scaffold *in-vivo* [S7 23].

f. Bone scaffold is dictated to induce new blood vessels, normally the healing wound after scaffold implantation *in-vivo* induce vascularization but this procedure takes weeks [S8 25]. The insufficient vascularization will cause oxygen and nutrients deficiency which endanger the culture MSCs causing non-uniform differentiation, and death of the cells [S9 28], incorporating VEGF in scaffolds could stimulate blood vessels growth.

Conclusion

As a conclusion that HA as tissue engineering scaffold material has many encouraging properties with minimal drawbacks which can be overcome in the future by creating composite scaffolding material using HA with certain fabrication criteria incorporated in organic or inorganic polymers, using the new advancement that we had in the polymerization technology to solve the HA drawback of weak biodegradability.

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Conflict of Interest

The authors declare no conflict of interest or personal circumstances that could be considered inappropriate that would affect the reported review by any means, and the funding sponsors had no role in the design of the study, collection or analysis of data and in the writing of manuscript or decision to publish the review.

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