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Multiscale Inorganic Hierarchically Materials: Towards an Improved Orthopaedic Regenerative Medicine

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Abstract: Bone is a biologically and structurally sophisticated multifunctional tissue. It dynamically responds to biochemical, mechanical and electrical clues by remodelling itself and accordingly the maximum strength and toughness are along the lines of the greatest applied stress. The challenge is to develop an orthopaedic biomaterial that imitates the micro- and nano-structural elements and compositions of bone to locally match the properties of the host tissue resulting in a biologically fixed implant. Looking for the ideal implant, the convergence of life and materials sciences occurs. Researchers in many different fields apply their expertise to improve implantable devices and regenerative medicine. Materials of all kinds, but especially hierarchical nano-materials, are being exploited. The application of nano-materials with hierarchical design to calcified tissue reconstructive medicine involve intricate systems including scaffolds with multifaceted shapes that provides temporary mechanical function; materials with nano-topography modifications that guarantee their integration to tissues and that possesses functionalized surfaces to transport biologic factors to stimulate tissue growth in a controlled, safe, and rapid manner. Furthermore materials that should degrade on a timeline coordinated to the time that takes the tissues regrow, are prepared. These implantable devices are multifunctional and for its construction they involve the use of precise strategically techniques together with specific material manufacturing processes that can be integrated to achieve in the design, the required multifunctionality. For such reasons, even though the idea of displacement from synthetic implants and tissue grafts to regenerative-medicine-based tissue reconstruction has been guaranteed for well over a decade, the reality has yet to emerge. In this paper, we examine the recent approaches to create enhanced bioactive materials. Their design and manufacturing procedures as well as the experiments to integrate them into engineer hierarchical inorganic materials for their practical application in calcified tissue reparation are evaluated.

Keywords: Bio-glass, bioactive scaffolds, bone, hydroxyapatite, templates, tissue engineering.

1. INTRODUCTION

By means of tissue engineering and tissue regeneration strategies, the objective of regenerative medicine is to restore diseased or damaged tissues to its original state and functionality, decreasing the need for transplants and joint replacements. The two approaches take the advantage of the utilization of scaffolds [1]. Fairly than just hosting cells into de diseased area to repopulate a defect and/or to return it function, in tissue engineering the cells are seeded in or onto biomaterials before transplantation. These materials act as provisional support and stimulate the reorganization of cells to recreate functional tissue [2]. Until now, it was supposed that scaffolds used in tissue engineering only served as a bracket. Extremely importance was placed on designing biocompatible and biodegradable materials with suitable mechanical properties and the exchange of cells into their structures. Nevertheless, as the field advanced, attention was focused on the biology of the scaffolds. Tissue engineers had recognized that extracellular matrix (ECM) is an active and a hierarchically ordered nano-composite that controls the essential cellular functions such as morphogenesis, differentiation, proliferation, adhesion and migration. The importance of the whole fibrillar and porous nanoscale topography of the ECM in stimulating essential cellular processes has led researchers to create biomimetic materials with nanoscale hierarchical features. The consequence of a scaffold nanostructured surface on the cells response is equivalent to other highly advanced strategies, such as the combination of synthetic ligands for cell adhesion receptors (e.g., Arg-Gly-Asp (RGD) or Lys-Arg-Ser-Arg (KRSR) amino acid sequences) onto the material surface, the assembly of three dimensional materials with controllable degradation rate, or controlled the release of growth factors and drugs from these materials [3].

The present article will explain why hierarchical structure is a key in the current trends of novel materials developments towards a superior orthopaedic regenerative medicine. It will then review the recent approaches to create enhanced bioactive glasses, ceramics and metals; the processing methods to tailor their structures, and why they have not been a commercial success despite of the acquired progress. At the same time the paper highlights the current progress on mate-

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rial-protein and material-cell dealings and the relevant place that occupies the hierarchical structure on such interactions. The purpose of this review is to provide the reader a general overview of hierarchical inorganic materials currently available for their theoretical or practical application in calcified tissue reparation. The examples that have caught our attention have been selected from a large literature collection.

2. WHY HIERARCHICAL MATERIALS

Structural hierarchy is a law of nature. Hierarchical structures can be observed in all bio-systems from chromosomes, proteins, cells, tissues, organisms and ecosystems. Owing to the even-increasing of variable environment and competition for survival, biological systems have evolved to optimize their functionality; and the resulting hierarchies in plants, animals and physiological systems are a logical manifestation of high efficiency and adaptability. Nature provides a huge amount of hierarchical biological materials with different functions [4-20], such as: abalone nacre [4, 5, and 20], crab exoskeleton [6-7], turtle shell [9], armadillo shell [10], spider silk [20], gecko feet [11-13, 20], cellulose [15-18], mineralized [21-24] and collagen tissues [24].

A number of decades ago, most of these biological materials were studied only by biologists. However, since the Material Science and Engineering (MSE) have been arisen in the 1950s, it has been an increased interest for biological materials [17, 25]. From the 1990s hierarchical nature structures have represented much attention due to their fascinating multi-functionality (self-organization, self-assembling, self-healing, self-cleaning, etc. [26, 27]). Current studies on biological materials have exposed that the typical size at each level of structural hierarchy may have been selected to ensure a specific function [28, 29]. One of the main driving forces in studying biological materials from the viewpoint of Materials Science is to employ the discovered natural organizations and methods as inspiration for developing innovative synthetic materials [30-40]. Replicating three-dimensional hierarchical structures for the purpose of repair or replacement of deteriorating tissues is one of the great challenges of chemistry, physics, biology and materials sciences [25]. It is therefore up to material scientists to explore, decode and understand the accumulated efficiency embedded in such systems, and to apply the obtained knowledge in their own benefit.

3. THE HIERARCHICAL STRUCTURE OF BONE

To be capable to regenerate new bone tissue, it is important to be aware of its structure. Bone is a natural hierarchically structured nano-composite of a protein based soft hydrogel template: collagen, non-collagenous proteins (laminin, fibronectin, vitronectin, etc.) and water; and hard inorganic components: hydroxyapatite, HA, $Ca_{10}(PO_4)_6(OH)_2$ [41]. Based on scanning and transmission electron microscopy [42] and by neutron scattering [43], it has been proposed that the lamellar structure is a very common motif in bone material. The organization in lamellar bone resembles to a rotated plywood structure, where the fibers are parallel within a thin sub-layer and where the fiber direction rotates around an axis perpendicular to the layers [44-46]. The origin of the rotated plywood assembly could be a twisted-nematic (or cholesteric) liquid crystalline array of collagen fibers [47]. Additionally to its evident biological value, bone has been well investigated by the materials engineering community because of its exceptional structure and mechanical properties. The hierarchical structure of bone has been described in a number of reviews [48-51]. 70% of the bone matrix is composed of HA nano-crystalline plates which are approximately between 25 nm in width, 35 nm in length and 2- 5 nm thick. Near 85-90% of the total bone protein consists of collagen fibrils [52-55].

More than 206 different bones make up the skeleton [56]; starting from the macroscopic structural level, they can have quite diverse hierarchical shapes depending on their respective function. Ranging from the long bones found in our limbs, such as the femur or the tibia, that provide stability against bending and buckling, to short bones in the wrist and ankle, and flat bones in the sternum and skull.

Bone is transformed, by the body, in response to its local loading environment. When a minor damage is done to a bone tissue, it can repair itself by the biochemical activity of the osteoblasts, called osteogenesis [55]. However, if the tissue's deficiencies exceed a critical extent, the bone cannot restore itself. Such defects can be a consequence of a trauma or from the elimination of unhealthy tissue. Graft implants (transplants) or synthetic bone filler materials are currently used to repair critical size bone defects. The purpose of regenerative medicine is to induce the body to reactivate osteogenic cells to re-create the natural 3D - bone architecture. Seemingly, the design of novel nano-materials possessing not only excellent mechanical properties but that are also biomimetic in terms of their hierarchical nanostructure, has become quite popular in order to increase bone cell functions and consequently the tissue regeneration.

4. APPROACHES TO CREATE ENHANCED MATERIALS.

A strong reason of why a perfect orthopaedic implantable material does not currently exist is, as we have described in a previous section, because bone is a structurally and biologically complex multifunctional tissue. The key matter to engineer the osseous regeneration process is to understand those factors that mediated the dialog between the bone cells and their microenvironment. The origin for this conception is that environmental biochemical and biophysical codes and signals are interpreted by cells into intracellular instructions that activate precise events in reaction to such signals and that make cells to adapt their behaviour to reply with the suitably response. Of the set of several factors that play a decisive roll in the tissue renewal process, it is essential to contemplate the following:

- Transference of oxygen, electrolytes, nutrients and molecules;
- Cell migration and mobility;
- Cell-materials interactions, where material surface characteristics have a direct and strong effect on cell performance;
- Biochemical and biophysical stimuli from the microenvironment;

− Control of angiogenesis;

− Control of immunological and inflammatory responses; and

− Cell behaviour, comprising attachment, proliferation and differentiation.

A scaffolding material can be used either to support bone cells or other biological agents, to release different active principles and, by the properly surface functionalization, to induce the growth of new bone from the neighbouring tissue [57].

4.1. Resources for inorganic multiscale hierarchical materials

There are many explanations of why implants fail: (i) reduced initial bone growth on the implant's surface; (ii) generation of wear debris in the implants' articulating components that become lodged between the implant and the surrounding tissue, leading to bone cell death, (iii) stress and strain imbalances between an implant and hosting tissue that leads to implant loosening and the eventual fracture. To overcome many of these problems bone implant materials need to:

a) be biocompatible;

b) form a chemical bond to host bone;

c) has an organized and interrelated pore structure to allow 3D bone in growth;

d) degrade (if possible) at identical velocity as the bone is repair;

e) has an appropriate surface for the attachment of osteogenic cells;

f) stimulate osseo-progenitor cells to produce bone extracellular matrix;

g) display mechanical properties comparable to that of the host bone;

h) be moulded by the surgeon to fit the correct tissue defect;

i) have the potential to be commercially producible and sterilizable for clinical use [57].

Scaffolds are short-term templates for bone growth and provide a specific environment and architecture for tissue development. In general, materials designed for this purpose are known as third-generation biomaterials, combining both biodegradability and bioactivity [58] and designed to trigger specific cell events at the molecular level. Some biodegradable polymers, ceramics and glasses have been explored and seem to match the criteria mentioned above. We also include metals, despite they are bio-inert and cannot well establish a tight and chemical bond with host bones, which easily cause subsequent wear and loose, thus ultimately resulting in implant failure; they posses excellent mechanical property, biocompatibility, corrosion resistance and high strength-to-weight ratio. For such reasons they have become most-used load bearing orthopaedic and dental implant materials in clinic practices [59].

4.1.1. Sol-Gel Bioactive Glasses

Bioactive glasses are constituted by an unsystematic network of silica tetrahedral containing Si-O-Si bonds. The network can be modified by addition of Ca, Na, and P atoms, which are bonded via non-bringing oxygen bonds. Phosphate ions also often are incorporated into the glass network, although it does not form part of the silica matrix, it forms orthophosphates balanced by calcium ions [57]. The history of bioactive glasses initiated in 1969, when Larry Hench opened a new research field by using glasses as implant materials [60, 61, 62]. Since then, this investigation line has provided very remarkable results in both academic and applied fields through the transformation of conventional glasses into glasses with biomedical added value [63]. Bioactive glasses link to and incorporate to the living bone without the formation of fibrous tissue around them or the promotion of inflammation or toxicity [64]. The extraordinary reactivity of these glasses is the central benefit for their utilization in periodontal repair and bone augmentation; the reaction products obtained from these types of glasses and the physiological fluids provoke the crystallization of the apatite-like phase, similar to the inorganic component of bones in vertebrate species. Furthermore, degradation ionic products, especially silica species, have revealed osteoinductive effects [65-67]. In summary, from a biological and chemical point of view, silica bioactive glasses display numerous of the properties related to a perfect material for grafting and scaffolding [68]. This feature endorsed new perspectives for SiO_2- based glasses as third-generation biomaterials for bone tissue regeneration [61]. Bioactive glasses can either be synthesized by melt; sol-gel and additive manufacture processes [68]. The original bioactive glass was melt-derived (46.1 mol% SiO_2, 24.4 mol% Na_2O, 26.9 mol% CaO and 2.6 mol% P_2O_5) and was named Bioglass®. At the beginning of 1990s bioactive glasses were created, for the first time, by the sol-gel process [69]. Bio-glasses exhibiting different porosities could be obtained from the hydrolysis and polymerization of metal hydroxides, alkoxides and/or inorganic salts. An extensive bibliography, comprising excellent reviews, summarizes this synthetic methodology and its application, enlightening how sol-gel chemistry provides a potential processing method for molecular and textural tailoring [70-76]. The authors of this review have explored different sol-gel methods to prepare SiO_2 glasses with hierarchical assemblies and bioactive characteristics. Based on a bottom-up reverse microemulsion method, it was shown the regulation of the stacking morphology and growth of the opal crystals with fibrous structure [77], Fig. (**1**). The development and the morphological adjustment of colloidal assemblies during a microemulsion-mediated hydrothermal synthesis is an intricate procedure which depends on the precise mish-mash of all microemulsion parameters. The influence of several elements on the absolute particle dimensions is essentially system specific, including the rate and the order of reactants combination. In the mentioned work [77], the microemulsion was considered as a pseudo-ternary system with oil, water, and surfactant constituents. The impact of microemulsion oil phase, the hydrothermal treatment time, and the calcination temperature on the final material organization were explored. In conclusion, two different materials created by opal micro-structured fibrils were produced; accordingly to the microemulsion template system, they exhibited both short and long wavelength light emissions and band gap values analogous to those found in silicon-based metal oxide semiconductors (MOS). Generally, the amalgamation of a

fibrous arrangement and photonic crystal properties just with a semiconductor structure runs a series of exceptional properties impossible to attain in classical silicon fibers. Reduction of materials at the nanometer scale provokes a number of unique optical and mechanical properties, including (1) large evanescent fields, (2) high nonlinearity, (3) strong confinement, and (4) low-loss interconnection to other optical fibers and fiberized constituents [78]. These evidences exposed an even superior range of possibilities for the applications of photonic crystal fiber (PCF). In these sense and thinking in the future use of Si-nanofibers as three-dimensional (3D) scaffolds for the creation of implantable artificial devices, the correlation among the material surface features and the amount, arrangement and organization of adsorbed fibrinogen (Fb) molecules was studied [79]. An improved understanding of the interaction among proteins and implantable materials' surfaces, particularly regarding to Fb, is essential for the knowledge of cellular events and the overall host response. These investigations have the potential to provide the foundation for the design of new materials where, the hemocompatibility is a key factor; a deeper description will be given in **section 3.1**.

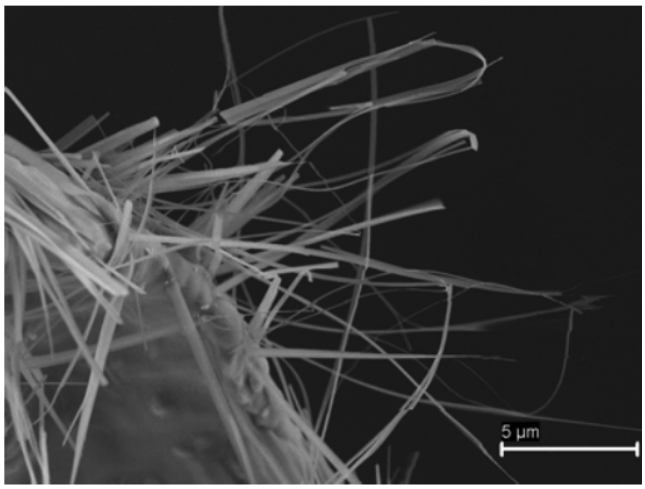

Fig. (1). SiO_2 nano-fibers; 30-50 nm diameter and more than 20µm length [77].

Since the trabecular bone exhibit sponge-like bicontinuity there is an increasing interest in the preparation of spongy-like sieves for the creation of bio-active implantable materials. The synthesis of bicontinuous pore silica materials by a one step sol-gel method using different bile salts' aqueous mixtures as templates was suggested as a simply and accurate strategy [80], Fig. (**2**). The effects of the molecular structure and the amount of bile salt on the synthesis progression are investigated and associated with the absolute material morphology. The regulation of such properties on the preparation of spongy-like silica materials and, its impacts on the material's bone-like apatite inducing ability in simulated body fluid (SBF) was evaluated. Under specific template conditions, it was possible to produce a bio-active open macro-mesopore structure material with a 3D-disordered sponge-like network comparable to that present in trabecular bone [80]. Contrarily to melt-derived bioglasses, sol-gel glasses are not prepared at high processing temperatures [70]. During the sol-gel process, the gelling stage occurs around room temperature. Gels, aerogels, glasses, dense oxides, and so on, can be made by sol-gel processing, thus facilitating the incorporation of organic and biological molecules within the network [81], or even cells within silica matrices [82]. Hassan [83] and Messina [84] *et al.* reported a facile method for the preparation of hierarchical silica nanostructures through a biomimetic approach based on Ovoalbumin [84] and Fibrinogen [83, 84] hydrogels as templates. The hydrogel matrix has tuneable physicochemical properties based on the thermal unfolding of the main domains of the protein. The network structures of the gels obtained are quite similar but differ in the mean pore size and rheological properties. These nanopores are then filled with silica precursors, under acidic conditions, where the condensation reaction is initiated. The protein hydrogel template is subsequently removed by calcination. The final materials show two different topologies. The origin of these two topologies lies on the anisotropic shape of the fibrinogen, driving stochastic interactions with the inorganic precursor and thus generating sponge-like (normal interactions) and polygonal fibers (parallel interactions) architectures. Sol-gel processes can be combined with supramolecular chemistry of surfactants, resulting in a new generation of highly ordered mesoporous materials for biomedical applications. Mesoporous materials have surface areas in the range of 600-1000 m^2g^{-1} and may accomplish the hierarchical requirements to be excellent candidates for controlled drug delivery systems [85] and bioactive materials [86]. These new glasses exhibit better bioactivity behaviour due to their outstanding values of surface area and porosity, as well as capability to host active agents that contribute to the tissue's healing processes. These characteristics make mesoporous SiO_2 excellent candidates to be used as implantable local drug delivery systems [87-89] and grafting materials for bone regeneration [90, 91]. In this strategy, the incorporation of structure-directing agents is essential for obtaining successful structures. Under appropriated synthesis conditions, these molecules self-organize into micelles. Micelles link the hydrolyzed silica precursors through the hydrophilic component and self-assembly to form an ordered mesophase. The mesophase ordering depends on several factors, such as sur-

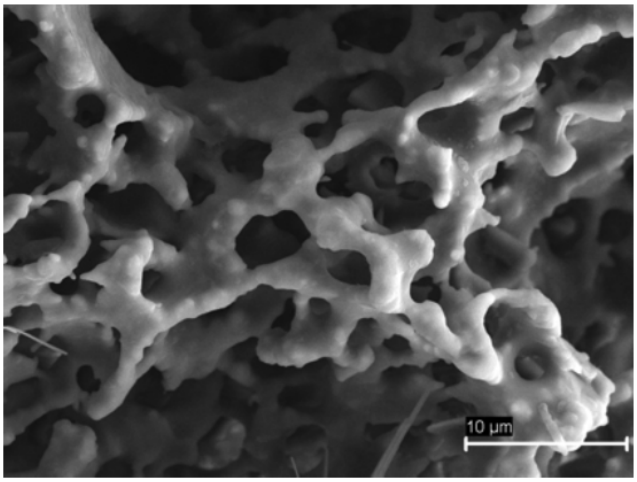

Fig. (2). SiO_2 spongy material templated by bile salts aqueous mixed systems [80].

factant chemistry (ionic, non-ionic, polymeric, etc.), organic/inorganic phase volume ratio, surfactant concentration, temperature, pH, etc. Once the product is dried and the surfactant removed (calcined or extracted with solvents), a mesoporous structure is obtained, exhibiting high surface area and porosity. It is not only bulk or monolith shapes that can be obtained by this method: inorganic and hybrid thin films are also obtained, with applications for many technological purposes as well coatings and membranes in the biomaterials field. The biodegradability of mesoporous membranes can be tailored with composition, porosity and calcination temperature, thus controlling the degradation timescale, which is especially relevant to the culture and growth of cells as well as for the design of drug delivery systems [92]. An extensive discussion on the synthesis conditions, processing parameters and methodologies can be found in reference [93]. Hyperthermia and local drug delivery have been proposed as potential therapeutic approaches for bone defects resulting from malignant bone tumors. The development of bioactive materials with magnetic and drug delivery properties may potentially meet this target. Wu and co-workers [94] build up a multifunctional mesoporous bioactive glass (MBG) scaffold system for both hyperthermic and local drug delivery applications. To this end iron (Fe)-containing MBG (Fe-MBG) scaffolds with a hierarchical large pores structure (300-500 μm) and fingerprint-like mesopores (4.5 nm) have been prepared. The magnetism of MBG scaffolds can be tailored by controlling the Fe content. Furthermore, the incorporation of Fe into mesoporous MBG glass scaffolds enhanced the mitochondrial activity and the expression of bone-related genes in human bone marrow mesenchymal stem cells (BMSC) attached to the scaffolds. The Fe-MBG scaffolds obtained also possessed high specific surface areas and demonstrated sustained drug delivery. Semiconductor nanocrystals and nanostructures have extensively studied in the last years due to their interesting optical and optoelectronic properties. Combining precise photoluminescence properties with controlled morphologies of SiO_2 is a major hurdle for a broad range of basic research and technological applications including the construction of biomedical devices. Ruso *et al.* [95] have demonstrated that microemulsion droplets interfacial elasticity can be manipulated to induce definite morphologies associated to specific intrinsic and extrinsic photoluminicent defects in the silica matrix. Thus, under precise experimental conditions hollow crystalline and compact amorphous SiO_2 spheres showing ultraviolet-photoluminescence while helicoidally fibrils of Ce-doped amorphous silica with violet-blue emissions are obtained, Fig. (**3**). Studies have shown that cerium has a positive effect on primary mouse osteoblasts *in vitro* and cerium oxide nanoparticles act as neuroprotective agents [96]. Overall, it is demonstrated that the combination of microemulsions and doping represents an easy strategy for the design of specific nanoscale structures with high efficiency photoluminescence. Other trace elements in the human body which plays an important role in bone growth and regeneration are incorporate to mesoporous bioactive glasses structures: Boron [97, 98], Zinc [96], Gallium [96] and Cobalt [98].

While direct foaming produces pore networks that mimic cancellous bone, control of pore size is limited to modal pore and interconnects sizes from the amount of surfactant used, the water content and agitation rate [70-76]. Pore morphology can be controlled more specifically using additive manufacturing techniques that can build scaffolds by depositing glass layer by layer [100]. The advantage of these techniques over foaming is that the scaffold pore structure is dictated by a computer-aided design (CAD) file. Recently, bioactive glass scaffolds were produced by a 3D printing process called ‘‘robocasting’’[101, 102]. The scaffolds produced had thick struts (>50 μm) and pores in excess of 500 μm, with 60% porosity. The alignment of the struts rows has a similar strength of cortical bone (>150 MPa were achieved in the direction of the pore channels; 50 MPa perpendiculars to the pore channel directions). A similar method, termed ‘‘freeze extrusion fabrication’’ (FEF), combines extrusion printing with freeze-drying. FEF was used to make glass scaffolds with 50% porosity and with pores and struts of equal size (300 μm), giving a compressive strength of 140 MPa [103, 104].

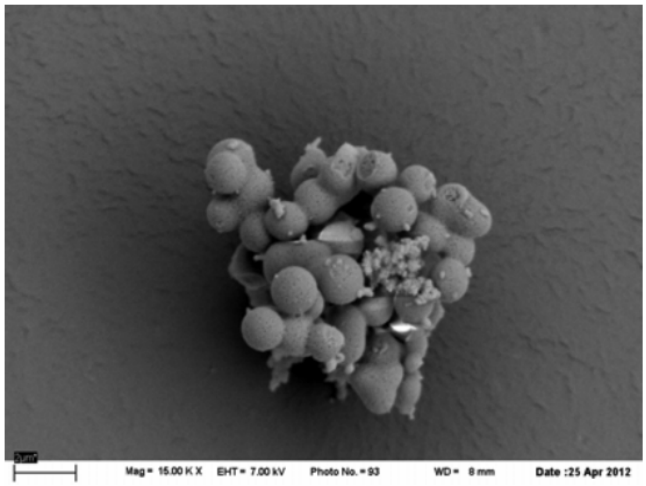

Fig. (3). SiO_2 spheres templated by reverse microemulsion systems [95].

4.1.2. Ceramics

Ceramics have been used as biomaterials for millennia. In fact in 1972, Amadeo Bobbio discovered Mayan skulls, some of then more than 4000 years old, in which missing teeth had been replaced by nacre substitutes [105]. Nacre is a natural composite consisting of 95-98 wt% of calcium carbonate (aragonite, the ‘ceramic’ phase) and 2-5 wt% of organic matter (fibrous proteins, polysaccharides). In clinical practice, the controlled implantation of ceramics started late 18th century in dentals with the use of porcelain for crowns and late 19th in orthopedics with the use of Plaster of Paris, or gypsum (calcium sulfate dihydrate) for bone filling [106]. With the advances in the ceramic technology, the 20th century saw more and more ‘high-tech’ ceramics available for medical purpose [107]. Tricalcium phosphate was first proposed in 1920 as a bioresorbable substance to fill bone gaps. However, tricalcium phosphate (TCP) and plaster are weak ceramics, unable to sustain significant loading. The need for tough and strong ceramics was not met before 1965, when the first alumina (Al_2O_3) material dedicated to hip joints was patented [108]. Synthetic calcium phosphate ceramics (together with calcium and/or phosphorus containing ceramics

and glasses) and zirconia were then proposed as alternatives to TCP and alumina, respectively. After roughly 100 years of clinical use, no tough and strong ceramic able to create a strong, biologically relevant interface with bone are created [109].

Numerous attempts based on calcium phosphate ceramic scaffolds have been made to mimic the structure of bone [110-112]. Even though their chemical composition, their only common point with bone is their porosity designed to allow bone ingrowth. They offer none of the structural feature of bone at smaller scales, and present no organic second phase. Porous calcium orthophosphate bioceramics with superior strength might be created from calcium orthophosphate fibers or whiskers. Fibrous porous materials are known to exhibit better strength and fracture deflection due to interlocking of the fibers. For example, porous bioceramics with well open pores was processed by sintering of fibrous hydroxyapatite (HA) particles. In another approach, porosity was achieved by firing apatite-fiber compacts mixed with carbon beads and agar. By varying compactaction pressure, firing temperature and carbon/HA ratio, total porosity was controlled in the range from 40 to 85% [113]. Recent developments in biomineralization [114] and biomaterials have demonstrated that nano-calcium phosphate particles play an important role in the formation of hard tissue in nature. It is suggested that the basic inorganic building block of bone and enamel are nanosized apatite although their hierarchical structures differ. Thus nanosized and nanocrystalline forms of calcium orthophosphates, Fig. (**4**), have great potential to revolutionize the hard tissue-engineering field, from bone repair and augmentation to controlled drug delivery systems. Nanoparticles can confer biominerals remarkable physical and chemical characteristics such as enhanced mechanical strength and self-preservation in biological fluids [115]. The use of nano-HA in orthopedics is considered very promising, due to its dimensional similarity with the bone crystals. Consequently, application and prospective use of the nanosized and nanocrystalline calcium orthophosphates for clinical repair of damaged bones and teeth are also well known [116]. For example, NanOss^{TM} bone void filler from Angstrom Medica Inc. [117] is considered to be the first nanotechonological medical device authorized by the US Food and Drug Administration (FDA) in 2005. It is prepared by the precipitation of calcium orthophosphate nanoparticles from aqueous solution, the resulting powder then being compressed and heated to form a dense, transparent material. NanOss^{TM} mimics the microstructure, composition and performance of human bone, and is mechanically strong and osteoconductive. It can be modeled according its medical application, so is used in the sport medicine, trauma, spine and general orthopedics markets [95]. Ostim® (Osartis GmbH & Co. KG, Obernburg, germany) is a ready-to use injectable bone matrix paste form based on synthetic nanocrystalline HA suspension that received CE (Conformite Europeenne) approval in 2002 [118]. The content of HA is about 35%, Ostim® does not harden off in contact with blood or spongiosa, so it is appropriate for autologous bone volume augmentation. At the same time, its viscosity enables it to be applied to form-fit in close contact with bone. It can be used in metaphyseal fractures, and cysts, acetabulum reconstruction and periprosthetic fractures during hip prosthesis exchange operations, oseteomies, filling cages in spinal column surgery, etc.[90, 93, 94, 119, 120]. It can be incorporated into bones, and new bone formation is visible 3 months later [121]. For a number of clinical applications Ostim® was combined with other types of calcium orthophosphates bioceramics, e.g., with HA bioceramice core (Cerabone®)[122] or with biphasic (HA + β-TCP) granules (BoneSaves®) [123]. Applications of such combinations appeared to be an effective method to treat tibia head compression fractures [96] and metaphyseal osseous volume defects in the metaphyseal spongiosa [100]. Also, they can be used in grafting procedures of acetabular bone [101]. Moreover, nanostructured calcium orthophosphates can be used as a coating material to impart surface bioactivity to other materials, for example glasses [124] or metals [125].

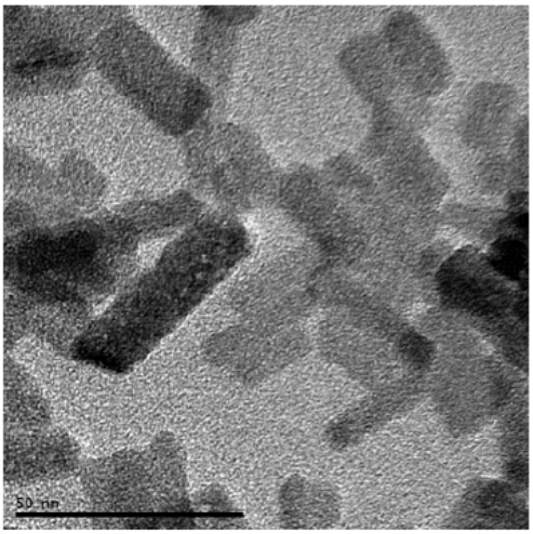

Fig. (4). Hydroxyapatite nanoparticles [127].

Actually, numerous attempts have been done to improve viability and proliferation of various types of cells on different crystalline calcium orthophosphates nano particles [126-130].

4.1.3. Metallic Scaffolds

Metals are extensively used in orthopaedics. The first metallic materials effectively used throughout the twentieth century in orthopedic applications were stainless steel and cobalt-chrome-based alloys. Ti and Ti alloys were introduced by the 1940s. NiTi shape memory alloys appeared by the 1960s and they seemed to open a whole new range of applications, due to their special mechanical behavior, but the properly unsolved allergenic effect of Ni has hampered their use [131]. Ti and its alloys, originally used in aeronautics, became materials of great interest in the biomedical field, due to their excellent properties that include a moderate elastic modulus of approximately 110 GPa, a good corrosion resistance and a low density (approx. 4700 kg m^{-3}) [131]. None of the metallic material used in orthopedics is bioactive *per se* and are inclined to form fibrous tissue at the metallic-bone interface, thus increasing the possibility of implantation loosening over a long period of time. Titanium metal itself is bio-inert, meaning that bone will not grow directly on untreated surfaces. Two approaches can be con-

sidered to obtain bioactive Ti. The first one consists of coating the surface of the implant with a bioactive ceramic (i.e., HA). The second one is to chemically modify the surface of the material so as to obtain the deposition of a bioactive ceramic *in vivo* or to induce proteins and cell adhesion and other tissue/material interactions. In the last case, the bioactivity starts off from the TiO_2 layer covering the metal surface. The induction of apatite layer nucleation and deposition on Ti surfaces was highly investigated. Bioactive Titania gels layers were induced by treatment of Ti substrates with a H_2O_2/HCl solution [132]. A similar effect was obtained by UV-light-illuminated TiO_2 powders [133]. In addition, Keshmiri and Troczynski [134] concluded that the particular surface morphology of the packed TiO_2 microspheres, promotes a faster apatite formation *in vitro*. The rough and termochemically treated Ti surfaces clearly induced a faster apatite deposition [135, 136]. Chen *et al.* [137] evaluate the effect of surface morphology, grain size, chemical composition, porosity and alkali-heat treatment on Ti powder and its ability to induce an apatite-layer deposition on its surface. The porous Ti samples and their nonporous counterparts made from powders with different mean particle size exhibited different surface energy values; reducing the Ti particle size increased the surface energy of the Ti samples and its ability to induce apatite deposition [137]. The Ti made from the smallest powders, with an average size of 5.89 ± 0.76 μm, yielded the highest surface energy (46.89 mJ m^{-2}). On contrary, no apatite was deposited on untreated Ti regardless of the particle size. Alkali-heat treatment increased the surface energy of Ti samples significantly compared to untreated samples; the apatite-inducing ability of the alkali-heat-treated porous Ti and their nonporous counterparts strongly depended on the surface energy. A hierarchically superhydrophilic structure on titanium surface with a nano-spongelike Titania layer on the micro-roughened titanium surface was constructed through dual acid etching and electrochemical treatments by Jiang and co-workers [138]. It was shown that the structure of micro/nano-spongelike TiO_2 not only provided better corrosion resistance and less oxygen vacancies, but also much higher ability of biomineralization after immersion in simulated body fluid (SBF) for 14 days. The micro-nano sponge-like structured surface on Ti significantly promoted human osteoblast-like MG63 cell attachment and proliferation. Fu *et al.* [139] reported a Ti-based, hierarchical porous scaffold anchored to Ti substrates, prepared by synthesizing hydroxyapatite calcium carbonate-Ti three layer spheres and combining a modified plasma spraying process and an anodic oxidation treatment. The porous scaffolds allowed good cellular and mechanical compatibility, high osteo-conductivity and osteo-inductivity, and strong osteo-integration.

Nanophase metals have been extensively investigated for orthopedic applications due to their higher surface roughness, energy, and presence of more particle boundaries at the surface compared with conventional micron metals. The first evidence that nanophase Ti, Ti6Al4V and CoCrMo significantly improved osteoblast adhesion compared to the respective conventional metals was provided by Webster *et al.* [140]

A surface modification strategy encircling the use of bioactive trace elements together with surface micron/ nano-topographical modifications was employed by Zhang *et al.* [141] in an attempt to enhance the osseointegration of Ti alloy (Ti-6Al-4V). The authors developed a strontium-substituted hardystonite (Sr-HT) ceramic coating with a hierarchical topography where the nanosized grains were superimposed in the micron-rough coating structure. Its ability to induce new bone formation was evaluated by an *in vivo* animal model (beagle dogs). Messina [142] and Ruso *et al.* [143] synthesized pure decahedral anatase TiO_2 particles with high content of reactive {001} facets by using a micro-emulsions droplet system as chemical microreactor and titanium (IV) tetra chloride ($TiCl_4$) as inorganic source, Fig. (**5**). The obtained cuboids around 90 nm in size have a uniform and dense surface morphology with a BET specific surface area of 11.91 m^2 g^{-1} and a band gap energy (2.30 eV) 1.39 times inferior to the anatase dominated by the less-reactive {101} surface (3.20 eV). The presence of reactive facets on Titania anatase favours the biomimetic growth of amorphous tricalcium phosphate after the first day of immersion in simulated human plasma demonstrating their bioactivity.

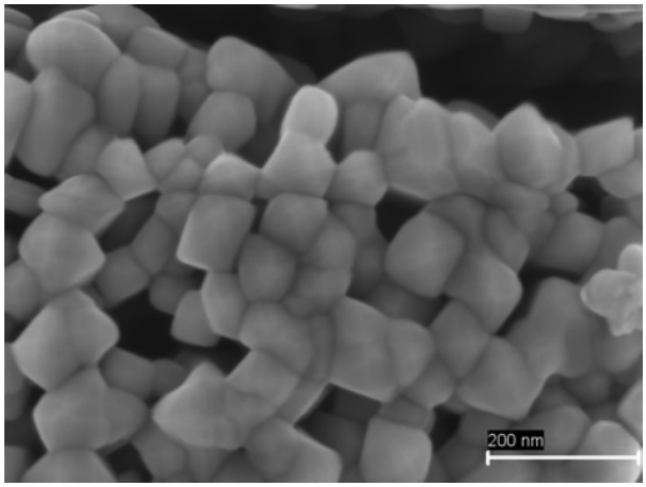

Fig. (5). TiO_2 cuboids nanoparticles [142, 143].

Controlling aligned fiber micro-architectures to simulate extracellular matrix inducing important biological functions is a key challenge in regard to successful tissue regeneration. Titania (TiO_2) nanotubes gained prominence as an implantation material due to its unique properties such as high specific surface area and the ability to exhibit positive cellular response [144-148]. The investigations of Puckett *et al.* [149] revealed that linear patterns of nano-features of Ti induced greater osteoblast adhesion than the micro-rough regions and also controlled osteoblast morphology and alignment. Yao *et al.* [150] informed about a greatly improved cell reaction on nanotubular anodized Ti. Wu *et al.* [151] demonstrated the fabrication of a hierarchical 3D microporous NiTi/Ti scaffold via a facile low temperature hydrothermal route. 1D nanotitanates grow naturally on the exposed wall of biocompatible 3D microporous NiTi/Ti scaffold in the form of nanowires and nanobelts on nanoskeletons resembling the lowest hierarchical organization level of human bone. More recently, Gravina *et al.* [152] presented a bottom-up microemulsion mediated strategy to obtain highly bioactive and biocompatible, striped Ce-TiO_2 nanocrystalline superstructures with $ONOO^-$ scavenging activity,

Fig. (**6**). The biocompatibility of materials was confirmed by assessing their interactions with neonatal rat calvarian osteoblasts. The increase of CeO_2 concentration and the effect of material micro-morphology on cell viability and adhesion were evaluated after 24, 48 and 72 h of treatment by microscopic observation. No significant changes were observed in cell adhesion or viability after 24 and 72 h of treatment in comparison with control. During the process of adhesion and spreading in the presence of material, osteoblast morphology was similar to control for all tested samples.

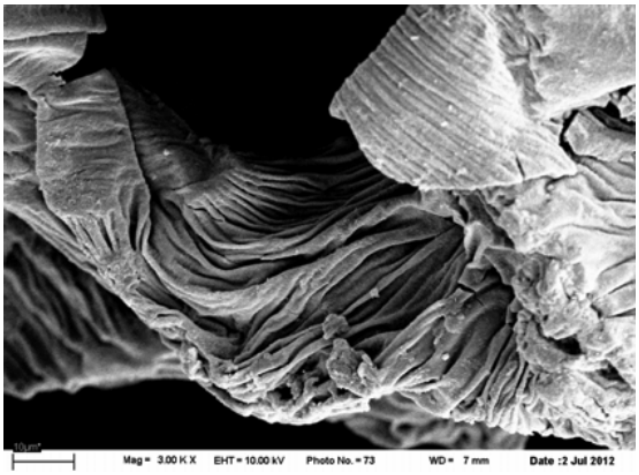

Fig. (6). Striped Ce-TiO_2 materials [152].

A relatively new class of nanomaterials, inorganic fullerene-like (IF) nanoparticles, have attracted much interest due to their potential applications in new medicinal technologies [153]. They have a hollow, stable and closed structure; usually produced from compounds with layered (2D) structures like MoS_2 [154, 155], WS_2 [156], BN [157] and $NiCl_2$ [158]. It was argued that, in analogy to carbon fullerenes, nanoparticles of such compounds suffer from inherent chemical instability in the planar form due to their abundant rim atoms. This chemical instability is alleviated by folding and the formation of seamless hollow structures, which are nevertheless elastically strained [153]. Recent results demonstrated a large spectrum of medical applications that could benefit from the superior tribological behavior of IF structures. A variety of medical devices has been coated and tested so far developing new clinical applications, including coatings for orthodontic wires [159, 160], catheters, heart valves, and stents [161]. Samorodnitzky-Naveh *et al.* [162] obtained stable and well-adhered cobalt plus IF-WS2 coatings on NiTi substrates. Friction tests presented up to 66% reduction of the friction coefficient. At light of the obtained results they have been suggesting that self-lubricating friction-reduction coatings based on MoS_2 and WS_2 nanoparticles with inorganic fullerene-like (IF) structure, can be used as coatings for artificial joints. Meanwhile, Tahir *et al.* [163] achieved the biofunctionalization of WS_2 nanostructures by the use of a polymeric ligand containing the tetradentate nitrilotriacetic acid (NTA) groups. The dual functionality of NTA permitted the anchoring of functional groups to the surface sulfur layer, and the linker groups for biofunctionalization through the immobilization of His-tagged protein (silicatein). They demonstrated that the protein retains its catalytic activity after the immobilization on the WS_2 nanotubes surface. Biofunctionalization of WS_2 nanostructures opens new opportunities for integrating this group of nanomaterials in composites, especially for immobilization of various proteins and biomolecules.

An important issue in biomaterial research have been the porous titanium implants. Porous structure is a way to minimize or eliminate the severe stress shielding to the tissue adjacent to the implant materials generated by the mismatch of Young's modulus between metals (i.e., 90-110 GPa for Ti and its alloys) and bone (0.3-30 GPa). A porous structure promotes osteo-integration and prevents implantation failure by providing spaces for bone cells, vascular and bone tissue ingrowth to form a mechanical interlocking. It has been proved that the optimal pore size for the cell attachment, differentiation and ingrowth of osteoblasts and vascularization is approximately 200-500 μm. Using a special powder metallurgy technique, Wen *et al.* [164] successfully fabricated a porous Ti with a porosity of 78% and a low Young's modulus (5.3 GPa) that exhibited a unique open-cellular porous structure. A summary of porous Ti scaffolds are presented in reference [165], including implants 50-60% porosity and 200-400 μm pore size coating for healing femoral defects in dogs [166]. Examples of completely porous metal scaffolds are titanium fibre meshes with 86% porosity [167] and 250 μm average pore size that have been used for the *ex vivo* culture of rat bone marrow stromal under static conditions or in a flow perfusion bioreactor [168] and subsequent implantation in cranial defects in rats [167, 168]. The First hierarchical titanium phosphate (TiPO) materials with multiple porosities of different lengths (meso-macroporous and meso-macro-macroporous) were synthesized by Ren and coworkers [169]. The formation of well-defined small macropore (50-160 nm in size) systems with mesoporous walls could be spontaneously acquired in the absence of any surfactant molecules and/or templates (such as colloid crystals and emulsion droplets), while the third tier of large macropores (500-1000 nm in size), in the form of parallel channels, could be originated from the participation of the poly-oxyethylene (POE)-surfactant molecules. Chu *et al.* [170] created a porous Titania structure with micro-isolated holes and grooves prepared by micro-arc oxidation on titanium; ZnO nanorods were subsequently electrodeposited on the walls of the pores to produce a micro-nano hierarchical structure suitable for biomedical applications.

4.2. Tailoring of the Structure

The interactions between cells and implantable materials will determine the success or failure of a medical device. Hierarchical topography is one of the most crucial physical cues for cells. So, as it was discussed previously, it is now being used in biomaterials science as a tool for controlling tissue regeneration. The ability to design hierarchical materials is thus essential for the development of new technologies and to improve existing ones. The goal is to choose molecular characteristics that will set the material properties on all length scales. A wide variety of techniques have been used to produce hierarchical topography with a regular controlled pattern on biomaterial surfaces. The production and assembly of hierarchical nano-topographies has recently been emerging very rapidly both by the optimization of current

methods and by the expansion of new nanoscience approaches. It is indispensable to focus on procedures that can create (i) extremely reproducible nanoscale topographies, (ii) surfaces with unchanging and rational nanostructures over large areas (in the range of cm^2), (iii) substrates with uniform surface chemistry and (iv) low production costs. Diverse tactics have been informed in the literature to fabricate 2D model surfaces; among them it can be mentioned: serial direct-writing photon and electron beam (e-beam) lithography, parallel replication, self-assembly nano-patterning, polymer de-mixing, molecular beam epitaxy and etching/precipitation. A briefly explanation and uses of these key methods are summarized in references [171] and [172]. Another reproducible, reliable, easy and fast approach to generate nanoscale surface topographies is by means of self-assembly procedures [173]; this methodology will be the focus in this review.

In many biomimetic processes, an inorganic layer is synthesized onto an ordered template, thereby producing the ordered hierarchical structure. A similar synthetic process can be developed using amphiphiles self assembly. Self-assembly is a biomimetic approach that can be applied to produce synthetic materials that resemble biological extracellular matrix [174]. Extracellular matrix scaffolds are crucial for skeletal-tissue regeneration. Amphiphilic molecules contain two distinct regions, one hydrophobic and one hydrophilic [175], and include surfactants (soaps), lipids and many block copolymers. In a selective solvent, such as water, amphiphilic molecules bring together into nanoscale aggregates such as micelles or bilayers. As a result, it is possible to control both its molecular properties, which determine the interactions between the cells and the scaffolding, and the spatial structure, which sets the new tissue-growth patterns. Amphiphilic nanoparticles become ordered in concentrated solutions to form'supramolecular' arrays. For example, spherical micelles form body-centred-cubic or face-centred-cubic assemblies, depending on the amphiphile's characteristics [176]. These supramolecular assemblies have been used as templates for the synthesis of highly ordered, hierarchical inorganic materials. First, an inorganic precursor (e.g.silicate) is used to coat the supramolecular assembly; the precursor then polymerizes to form an inorganic phase whose structure is set by the underlying amphiphile template. This method has been successfully used to produce a variety of structures with hexagonal, cubic and lamellar symmetries [177]. More recently it was applied to generate hierarchical porous films formed by interconnected pores of different dimension [178]. A similar approach uses monodisperse particles such as colloidal spheres [80, 127] or emulsion droplets [77, 79, 95, 142, 143, and 152] for templating. The particles self-assemble into an ordered array that acts as a base for the inorganic lattice. This method has produced highly ordered structures in a range of geometries. In both types of process, the organic scaffolding can be removed after the inorganic structure has been set, by heat treatment or by dissolution with a solvent to leave a periodic array of pores. In nature, helical macromolecules such as collagen, chitin and cellulose are critical to the morphogenesis and functionality of various hierarchically structured materials. These macromolecules undergo self-templating assembly, a process whereby multiple kinetic factors influence the assembly of the incoming building blocks to produce non-equilibrium structures [179]. Proteins and other biosystems can be used as templates of hierarchical materials [180]; critical reviews were presented by Stephanopoulos *et al.* [180, 181]. Other examples include the use of Fibrinogen and Ovoalbumin hydrogels as templates of spongy bioglasses [83, 84] and the preparation of hierarchical bimodal porous structured mesoporous bioactive glass (MBG) materials [182] using a bio-templated synthesis based on the natural Mediterranean Sea sponge as macropored hard templates and the surfactant Pluronic (P123) as mesopore soft templates respectively.

Unidirectional freezing and ice-segregation-induced self-assembly (ISISA) is another technique for the preparation of materials with highly sophisticated structures (e.g., hierarchical materials exhibiting organization at different scale levels) [183]. Cryogenic processes (consisting of the freezing, storage in the frozen state for a definite time, and defrosting of low - or high-molecular-weight precursors, as well as colloid systems, as either a water solution or suspension, or forming a hydrogel) have been widely used for the scaffolds preparation. Gutiérrez *et al.* [184] exemplified how the aqueous nature of the ISISA process allows for the in-situ incorporation of biological entities which provides not only hierarchy but also functionality to the resulting materials. Interesting examples of biocatalytic materials (for organic synthesis and fuel cell technologies) and biosensors, and scaffolds exhibiting enhanced functional (in terms of both biocompatibility and biodegradability) and mechanical performance, are reviewed in their work.

5. BIOCOMPATIBILITY OF ENGINEERED STRUCTURES.

Being a dynamic tissue, the bone responds to biochemical, mechanical and electric signals being remodelled it so that maximum strength and toughness are along the lines of the greatest applied stress.

5.1. Interaction with Soluble Proteins

When biological systems approach into contact with biomaterials, for example by grafting in the human body, proteins naturally adsorb onto the surface. The statement of that nanoscale surface topography, in the absence of serum, does not considerably affect cell adhesion [185], is a fascinating finding that highlights the significant role of adsorbed proteins on the modulation of cellular communications. The proteins' orientation and conformation once they are adsorbed onto surfaces is critical for cell integrins to recognise the specific sites which may initiate the signalling events. The resulting surface-bound protein layer arbitrates the subsequent cell attachment through interactions with cell's surface receptors [186, 187]. Consequently, the geometrical and chemical properties of biomaterial surfaces direct cellular functions such as cell attachment, proliferation, migration, differentiation and modify their receptiveness to extracellular signals. As the cellular response to nanoscale surface topography is governed by the adsorbed protein layer, the upcoming strategies focusing on nanomaterials should enhance the activity and selectivity of protein adsorption.

Commonly, it has been revealed that nanoscale surface topography significantly influences both in the protein adsorption and in the cellular response [186, 188-190], making it a key factor in the interactions between biological systems and artificial interfaces. This is to be predictable as *in vivo* cells respond to textured signals in the ECM [191], which has pits, pores, protrusions and fibers in the scale range of 5 - 200 nm [186]. Nanotopography, thus, may offer biomimetic's cell-modulating cues to biological units [192]. Several reviews that centre on the physicochemical aspects of the adsorption of proteins to solid surfaces, and on the biological screening methods designed to further explore the impact of nanotopography, are available [186-191, 193]. Information on how the surface topography affects the protein adsorption is varied due to the many ways of nanoscale surface topographies that can be created [186-191, 193]. Dynamic interactions between nanoscale surface topographies and proteins are intricate due to the combination of attractive and repulsive forces that are governed by local changes in surface properties, including chemistry, which may lead to three-dimensional (3D) changes in the quantity, density and orientation of adsorbed proteins. Alterations in the selectivity, as well as the quantity, of proteins adsorbed from serum onto stochastically nano-rough surfaces, display that increased surface area in combination with another mechanism must be responsible for the different protein adsorption on nanoscale surface structures [194]. Surface roughness has been stated to disturb the surfaces' wettability, leading to localized changes in superficial chemistry or limiting the protein exchange on the surface. Furthermore, the protein anisotropy, in combination with the surface nano-topography may induce diverse degrees of geometrical packing of the proteins [79, 195], while the protein's concentration is also found to impact on the amount and the adsorbed proteins' conformation [79, 196].

The complete effect of nanoscale surface's topography on protein adsorption is not clear from the reports currently available in the literature. Numerous revisions showed that there is a no linear relationship between protein adsorption and surface roughness. Cai *et al.* [197], establish that the amount of fibrinogen adsorbed onto stochastically nano-rough titanium surfaces was unchanged in comparison with flat control titanium. In addition, studies on Poly(ether sulfone) ultra filtration membranes with increasing pore sizes from 5 to 60 nm exposed to hen egg lysozyme [198] presented that, at these scales length, no noteworthy changes in the secondary structure of egg lysozyme occurred with increasing surface roughness. Moreover, no distinctive difference in the adsorbed amount was observed with increasing surface roughness. These findings were endorsed to the fact that the overall size of the surface available for adsorption was significantly superior to protein's size. It was further supposed that surface roughness only becomes important when the overall size of the surface feature is within the protein's dimensions range. On the contrary, the amount of fibrinogen on nanostructured surfaces has been shown to be transformed with dissimilar stochastic roughnesses using colloidal silica particles adsorbed onto a polycationic polymer [199]. Rechendorff *et al.* [200] display that the amount of fibrinogen on tantalum surfaces used for grafts was directly influenced by the surface nano-roughness. Alternative studies investigated the influence of surface nano-roughness on the adsorption of bovine serum albumin and streptavidin [201]. It was exposed that an intensification of the nanoscale surface roughness of TiOx surfaces films from 15 to 30 nm causes a decrease in protein binding affinity and an increase in the amount of adsorbed protein. Gold nanoparticles with a diameter of 150 nm were assembled onto smooth gold substrates and then the amount of adsorbed serum proteins was compared to smooth gold surfaces, in order to evaluate the influence of nanostructures on the activation of the immune complement system [202]. It was shown that the nanostructured surfaces led to a significant increase in serum adsorption of up to 70%. Nevertheless, the activation of the immune complement was drastically reduced by nanostructured surfaces compared to their smooth counterparts. Hassan *et al.* [79] assessed the adsorption process of fibrinogen (Fb) onto Opals fibrils. The whole Fb adsorption process is irreversible, with a high alteration of the initial material morphology. The equilibrium value for the adsorbed Fb surface concentration is about (270 ± 20) μg dm^{-2}. The examination of the material morphology before and after Fb adsorption by scanning electron microscopy, revealed that after protein interaction the original fibrous structure was disrupted, giving rise to fibers of higher dimensions or a bicontinuous structure. The fibrous structure and the similitude in size between the fibrous substrate (d = 30-50 nm) and the Fb molecules (47-50 nm), is suggested to be the key to the improved adsorption progression. After adsorption, the αC-domains are seemingly available for interaction with the domains of the neighbouring adsorbed Fb molecules, thereby stimulating lateral and equilateral associations and the creation of an extensive network similar to those that exist in nature between fibrin units.

From the evidence examined above it is unclear the relationship between protein adsorption and surface roughness leading the need of further investigation. At present, the influence that surface topography has on protein adsorption and particularly on protein unfolding has to be scanned for each situation. It remains challenging to predict a general behavior, particularly in complex mixtures.

5.2. Interaction with Cells

The guidance of nano-topography on cellular response has been explored using a variety of surface structures and cell types including osteoblasts [127, 152, 186, 203], fibroblasts [186, 204], macrophages [185, 205], neural [206, 207] and endothelial cells [186, 204]. Some selected examples were stated below. Zhang *et al.* [208] set three types of Ca-P ceramics with different Ca-P ratios, i.e. HA, beta-tricalcium phosphate (-TCP), and biphasic calcium phosphate (BCP) ceramics with dense-smooth and porous structures. An exhaustive gene expression microarray analysis of mouse osteoblast-like cells cultured on the presence of these materials exposed that porous Ca-P ceramics have considerably affected the gene expression profiles, having a higher potential for osteoblast maturation. In the subsequent *in vivo* study performed by the authors, alkaline-phosphatase-positive cells were detected in the pores of hydroxyapatite and BCP, and the expression of the osteocalcin gene (an osteoblast-specific marker) in tissue grown in pores was also superior in hydroxyapatite and BCP than in -TCP. It was detected the ex-

pressions of marker genes of the early differentiation stage of chondrocytes and the complete differentiation stage of adipocytes (originate from mesenchymal stem cells, as well as osteoblasts) in the pores of any Ca-P ceramics, 16 weeks after the implantation. These marker gene expressions were not detected in the muscle tissue surrounding the implanted Ca-P ceramics. Nano-hydroxyapatite particles have better bioactivity than the coarse crystals. So, they can be utilized for engineered tissue implants with improved efficiency over other materials. D'Elía *et al.* [127] presented a study that involves different hexadecyl trimethyl ammonium bromide (CTAB) micellar - block copolymer organized networks to create bioactive superstructures resulting from HA nano-rods associations. At the synthesis conditions CTAB form rod-like micelles of 47 nm length [209] that template the deposition of PO_4^{-3} and Ca^{2+} ions favoring the formation of bone dimensioned HA nano-rods. The interaction with block co-polymers restrings the crystals growth and directs their association inducing the final structure arrangement. The structure set-up and evolution relies on a synergistic effect of the mutual interactions of the confined reaction media environment (block copolymer network) in contact with the external template (CTAB rod-like micelles). The manipulation of such interactions permits the alteration of the chemical and/or the surface properties on the synthesized materials and their subsequent bioactivity, including mineral coating morphology and growth kinetic. The proposed method let us to obtain bioactive and biocompatible materials which may allow replicate in some extent the bone structure, HA nano-rods of 25-50 nm length organized in hierarchical structures. The biocompatibility of materials was confirmed by assessing their interaction with neonatal rat calvarial osteoblasts; viability and cell adhesion were evaluated after 24, 48 and 72h of treatment by microscopic observation. When osteoblasts were seeded in the presence of the material (T1), after 24 h they showed adherence to the culture plate in a similar manner to the control cells. In a second procedure the cells were plated and 24 h latter the material was added (T2); in such conditions no significant changes were observed in the cell adhesion after 48 and 72 h of treatment compared with control and with T1. During the process of adhesion and spreading in the presence of material, the cell behavior was similar to control in both treatments; they showed their typical polygonal or widespread forms with fine filopodia and abundant surface folds. No significant differences in cell viability after 72 h of both treatments (T1 and T2) with respect to the controls were observed (control: 94.21 ± 4.2%; T1: 97.62± 5.62%; T2: 98.72 ± 4.92%).

Regulate the aligned fibers' micro-architectures to mimic the extracellular matrix and induce important biological functions are a key challenge in regard to successful tissue regeneration. Gravina *et al.*[152], described the use of a simply bottom-up reverse microemulsion method to manipulate the morphology of Ce doped TiO_2 crystals during an hydrothermal synthesis at 100ºC. Phase behavior measurements were conducted on several systems to identify the properly synthesis conditions. Under precise synthesis circumstances a striped Ce-doped TiO_2 material was found. The material was planned to merge fibrillar nanoscale morphology and the scavenging properties of Ce on a TiO_2 substrate. As it was mentioned above, the role of the materials surface properties in cell guidance are well known, but how these aspects affect their bioactivity is still under investigation. Thus, the authors have examined the effect of surface area, morphology and roughness on the material bioactivity and biocompatibility. The presence of Ce atom in TiO_2 crystalline lattice stabilized anatasa polymorph, and such fact had a noticeable influence on the material bioactivity. The epitaxial growth of HA is preferentially on (110) anatase surface and plate-like nano-morphology crystallites of ~ 150 nm length arranged in spherical-like globules with 3-5 μm in diameter. Such morphology, according to literature, is essential for bone-bonding. The biocompatibility of TiO_2-CeO_2 materials was established by evaluating their interactions with neonatal rat calvarian osteoblasts. The increase of CeO_2 concentration and the effect of material micro-morphology on cell viability and adhesion were evaluated after 24, 48 and 72h of treatment by microscopic observation. No significant changes were observed in cell adhesion, viability and morphology after 24 and 72 h of treatment in comparison with control and with the material without ceria. Similar results were obtained varying the material micro-structure. The attained results eliminate any negative effect of the material crystalline microstucture, morphology and CeO_2 presence in the osteoblast survival.

We have previously described the possibility to nanostructural features of material surfaces have been shown capable to alter the 3D conformation of adsorbed proteins and this could potentially have an effect also on host adhesins filming the biomaterial surfaces. Based on current evidence, it is possible to produce antifouling surfaces by acting on the nanotopology, reducing the area available for bacterial attachment or generating superhydrophobic surfaces. Nanostructure appears to go far beyond simply contrasting bacterial adhesion, the nano-pillar arrays present on the wing surface can even induce bacterial cell death [205].

A lot of investigations have confirmed that nano-porous structure in bioactive glasses can remarkably improve their bioactivity. Nevertheless, researches on preparation of nano-porous bioactive glasses in the form of film coating and their cell response activities are scarce. Ma *et al.* [210] prepared and analysed the *in vitro* bioactivity of a nano-porous bioactive glass film (nBGF) on commercial glass slide based on a sol-gel technique. Cell responses of the samples, including attachment, proliferation and osteogenic differentiation, were also examined using BMSCS (bone marrow derived mesenchymal stem cells) as a model. The nBGF showed an asymmetrical but homogeneous porous structure with large specific surface area and pore volume. Introduction of such nano-porous structure profoundly accelerated the deposition of HCA on nBGF, and at the same time promoted the proliferation of BMSCs, indicating remarkable increase of the biocompatibility. Meanwhile, BMSCs incubated on nBGF attached to the film more tightly and exhibited more plump and stereo morphology with larger number of synapses. Midha *et al.* [211] evaluated the potential of a sol-gel-derived bio-glasses 70S30C (70% SiO_2, 30% CaO) BG foams as a candidate scaffold for bone regeneration by investigating its osseo regenerative potential in a rat tibia defect model. Bone ingrowth into the preconditioned scaffolds was similar to that in NovaBone (melt-derived Bioglass par-

ticles); Actifuse (Si-HA porous granules). Nevertheless the Actifuse and NovaBone granules showed little evidence of material degradation and also intruded into adjacent marrow cavities, causing ectopic bone formation. The benefits of the preconditioned 70S30C scaffolds include: the defect-specific 3-D geometry kept them in position for the 11 week period; bone formation did not encroach the marrow cavity; and enhanced degradation of the scaffold promoted bone regeneration within the dimensions of the tibia shaft.

Hence, generally trends of the effect of nano-topography on cellular response are difficult to determine and are quite cell type and application specific. Through comparison of many studies reporting the effect of nano-topography on cellular response is hampered by differences in the methods of cellular characterization and the topographic dimensions and chemistry of the nanoscale surface features. Despite of this, the general trend is that nanoscale surface topographies are capable of, in the short and medium term, modulating cellular responses.

CONCLUSION AND OUTLOOKS

A recognized route to promote tissue regeneration in reconstructive orthopaedics is the employ of scaffold that can simultaneously address both mechanical and biological bone constraints. The scaffold's micro-architecture must be specifically tailored to locally match belongings of the host tissue resulting in a biologically fixed implant. As such, the use of an optimized graded scaffold mitigates otherwise present issues causing implant failure such as bone resorption and shear stress at the interface.

Hierarchical structures refer to the fact that features at scales from the nanometre to millimetre level will determine how well the scaffold ensures tolerance of material/ structural flaws. They are particularly attractive for the construction of bone-replacement materials, since hard tissues are hierarchically organized and exhibit order on multiple length scale, from the nano to the macro level. Advances in bone substitute scaffold design must enable hierarchical structures to attain desired mechanical function and mass transport. These approaches, located at the interdisciplinary area between biomedical materials science and curative medicine, will become more affordable by combining with biomimetism, which not only optimizes biomaterial interaction with biological tissues but also mimics biogenic materials in their configuration and functionalities. We consider that hierarchically designed materials can be applied to create the next generation of tissue engineering scaffolds. Structural heterogeneity and hierarchy are inherent features in biological nature materials; so those characteristics have a great potential for findings new solutions of regenerative medicine problems.

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