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Gisela M. Luz, João F. Mano. Mineralized structures in Nature: examples and inspirations for the design of new composite materials and biomaterials. *Composites Science and Technology*, 2010, 70 (13), pp.1777. 10.1016/j.compscitech.2010.05.013 . hal-00681646

HAL Id: hal-00681646

<https://hal.science/hal-00681646>

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Accepted Manuscript

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PII: S0266-3538(10)00197-1
DOI: [10.1016/j.compscitech.2010.05.013](https://doi.org/10.1016/j.compscitech.2010.05.013)
Reference: CSTE 4718

To appear in: *Composites Science and Technology*

Received Date: 25 December 2009
Revised Date: 30 April 2010
Accepted Date: 11 May 2010

Please cite this article as: Luz, G.M., Mano, J.F., Mineralized structures in Nature: examples and inspirations for the design of new composite materials and biomaterials, *Composites Science and Technology* (2010), doi: [10.1016/j.compscitech.2010.05.013](https://doi.org/10.1016/j.compscitech.2010.05.013)

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Mineralized structures in Nature: examples and inspirations for the design of new composite materials and biomaterials

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Abstract

Through the natural evolutionary process, organisms have been improving amazing mineralized materials for a series of functions using a relatively few constituent elements. Biomineralization has been widely studied in the last years. It is important to understand how minerals are produced by organisms and also their structure and the corresponding relationship with the properties and function. Moreover, one can look at minerals as a tool that could be used to develop high performance materials, through design inspiration and to find novel processing routes functioning at mild conditions of temperature, pressure and solvent type. As important as the molecular constituents are structural factors, which include the existence of different levels of organization and controlled orientation. Moreover, the way how the hierarchical levels are linked and interfacial features plays also a major role in the final behavior of the biogenic composite. The main aim of this work is to review the latest contributions that have been reported on composite materials produced in nature, and to relate their structures at different length scales to their main functions and properties. There is also an interest in developing new biomimetic procedures that could induce the production of calcium phosphate coatings, similar to bone apatite in substrates for biomedical applications, namely in orthopedic implants and scaffolds for tissue engineering and regenerative medicine; this topic will be also addressed. Finally, we also review the latest proposed approaches to develop novel synthetic materials and coatings inspired from natural-based nanocomposites.

Keywords: Biomimetics, biocomposites, structure-property relationships, biomaterials, mineralization.

1. Introduction

Nature, through the evolutionary process, has been able to design and produce highly sophisticated materials, used for a variety of functions, including for structural purposes [1], [2]. The physical properties of biological systems, such as the mechanical performance, are typically far better than that of the equivalent synthetic materials, with similar compositions and processed with present technologies. Moreover, these materials are produced at mild temperature and pressure conditions, with relatively low energy consumption. Finally, such systems are made with significant weak components such as brittle minerals, soft proteins and water. Therefore, nature has been a fascinating source of inspiration for scientists and engineers. Biomimetics is an emerging field of science that includes the study of how Nature designs, processes and assembles/disassembles molecular building blocks to fabricate high performance hard polymer-based composites (e.g., mollusc shells, bone, tooth) and/or soft materials (e.g., skin, cartilage, tendons), and then applies these designs and processes to engineer new molecules and materials with unique properties [3], [4], [5]. Biologically inspired design or adaptation or derivation from nature is referred to as 'biomimetics'. It means mimicking biology or nature. The field of biomimetics is highly interdisciplinary. It involves the understanding of biological functions, structures and principles of various objects found in nature by biologists, physicists, chemists and material scientists, and the design and fabrication of various materials and devices of commercial interest by engineers, material scientists, chemists and others [6].

Mineralized biological materials are a subgroup of this immense world that have fascinated many researchers due to their unique structure and performance. Despite the large variety of existing biocomposites, only about 60 minerals are used by organisms, approximately half of them containing calcium [7], [8]. It has been recognized that some constituents, such as calcium carbonate or silica, exist recurrently in very different mineralized organizations and processed by completely diverse living organisms. As important as the molecular constituents are structural factors, which include the existence of different levels of organization and controlled orientation. Moreover, the way how the hierarchical levels are connected also plays a major role in the final behavior of the biogenic composite. The main aim of this work is to review the latest contributions that have been reported on natural mineralized materials, and to relate their structures at different length scales (molecular, nanometric, micrometric and macroscopic levels) to their main functions and properties. An emphasis will be on discussing topics that include how the different hierarchical levels are organized and what would be the contribution of both the organic fraction and water that exists within such structures. A series of examples of mineralized structures will be presented, including the fracture surface of shells, bone and teeth. The study of the structure-function relationships in bone has been particularly investigated, together with studies in the area of calcium phosphates, as hydroxyapatite constitutes the inorganic component in this tissue. In this context, much work has been done in the development of new procedures that could induce the production of calcium phosphate coatings in substrates for biomedical applications, namely in orthopedic implants

and tissue engineering scaffolds; this topic will be also reviewed. Finally, we also review the latest proposed approaches to develop novel synthetic materials and coatings inspired from natural-based composites. The difficulties associated with such biomimetic routes will be pointed out, related to the complex architecture found in the biogenic composites and with interfacial arguments. In fact, many aspects of biomineralization are far from being clearly understood and suggestions on how such fundamental knowledge could be transposed to useful synthetic strategies for developing completely new materials offers a series of stimulating research opportunities for young researchers.

2. Structure of mineralized biocomposites and properties relationships

2.1. General considerations

It has been recognized that biological composites exhibit a series of organized structures on discrete scale levels, ranging from the molecular to the macroscopic [9], [10]. The components at each level are interrelated with each other such that the performance for the required functions can be optimized. The different levels are assembled through a bottom-up approach using nanofabrication methodologies, mediated by cellular signals. It is interesting to notice that the hard mineral component in natural composites exhibit nanometric sizes, at least in one direction, displays an anisotropic geometry and is immersed in a soft organic matrix. For example (see more details later), enamel is composed by 15-20 nm thick and 1000 nm long crystals, with low soft protein matrix content; dentin and bone contains plate-like crystals (2-4 nm thick) and double the quantity of protein matrix; nacre is made of plate-like crystals (200-500 nm thick) and contains a very small amount of soft matrix. In a pure fracture-mechanics perspective Gao and co-workers showed that nanometer scale plays a key role in allowing these biological systems to achieve their superior mechanical properties [11], [12]. Below a critical size on the nanometer length scale, the mineral crystals fail no longer by propagation of pre-existing cracks, but by uniform rupture near their limiting strength. This increase of robustness is also achieved through the hierarchical organization, as studied by modeling self-similar composite structures mimicking the nanostructure of bone. Such mechanistic analysis also allowed the understanding the importance of the anisotropic structure of the mineral crystals that can explain how nature can produce stiff composites with low mineral content (e.g. bone): it was shown that the large aspect ratio of mineral crystals in bone can fully for the softness of the matrix [12].

Another important aspect in the common structure of many mineralized biological materials, namely the ones that were produced to fulfill a multipurpose function (e.g. mollusc shells, skeleton of sea urchins, lamellar bone and biogenic silica) is that they seem to be designed to reduce the extent of mechanical anisotropy [13]. Here, the idea of multipurpose material is related to material produced by a variety of different organisms of the same taxon, and used in anatomically different environment. Therefore, they should function under many different situations and should respond mechanically more or less

equally from all different directions. In these systems one may observe highly anisotropic structures at some hierarchical level (for example, the mineralized fibrils within the layers of lamellar bone), but it seems that the design strategy throughout the entire levels of structure is to enhance, as much as possible, the isotropic character of the material [13].

An interesting example of structural hierarchy was reported for a silica-based mineralized skeleton of a sponge, providing a unique example of how glass, a classic brittle material, can be used as a structural element in the biological world [14]. The assembly towards the glassy cage of this organism involves at least seven hierarchical levels, all contributing to mechanical performance. Silica nanospheres are arranged into concentric layers, held together by organic layers, to yield lamellar fibers. Those are organized in bundles to produce flexural rigid composite microscopic beams. The beams are arranged in a rectangular lattice that, together with other structural motifs, provides adequate mechanical features of the glass skeletal system at the macroscopic scale. Some more examples of mineralized biocomposites will be given, where the relationship between structure and performance or function will be highlighted. As the main applications of nature inspired synthetic materials that we intended to focus are in the biomedical field, a special attention will be given to human mineralized tissues (bone and teeth). Shells will be also discussed as calcium carbonate-rich nanocomposites as they also exhibit particular structural features and properties that can also be interesting in the orthopedic field.

2.2. Bone

Bone refers to a family of materials that all have the mineralized collagen fibril as their basic building block [15]. The general structure of bone is summarized on figure 1 (A). Plate-shaped crystals, with 50x25 nm of length and width and with 2-3 nm thickness, of carbonated hydroxyapatite, with crystals aligned along their c-axis, are embedded in a type-I collagen framework. The fibrils consist of triple-helix collagen chains with 1.5 nm diameter and 300 nm length. Their ends are separated by holes of ca. 35 nm and the neighboring molecules are vertically offset by 68 nm. The apatite crystals are nucleated at specific regions on or within the collagen fibrils. They grow in the hole zones that exists between neighboring collagen molecules. For the particular case of lamellar bone the fibrils are then arranged in parallel arrays, with crystals aligned (sub-layers). The consecutive sub-layers rotate through the lamellar plane by an average of 30°, forming a so-called plywood-like structure. As each lamella is composed of five sub-layers, the total rotation is 150°, thus forming an asymmetric structure. Moreover, the collagen fibril bundles rotate around their axis within the five sub-layers. Both facts enhance the isotropic properties of bone found at the macroscopic scale, as previously reported. Moreover, this type of architecture hinders crack propagation and increases toughness. Figure 1 (B) displays a typical lamellar morphology found in cortical bone, showing a cryogenic fracture surface of a bovine femur. The layered texture reveals the complex and periodic structure of the lamellar bone, being in agreement of the plywood-like structure. The lamellae that form cortical bone have three forms of appearance: the secondary osteons, interstitial lamellae and the inner and

outer circumferential lamellae. The secondary osteons are cylindrical tubes, which are permanently re-built by the remodeling process. The remodeling is regulated by the mineral metabolism and the appearance of microcracks caused by fatigue. Osteoblasts destroy the bone tissue, forming a tunnel along the longitudinal axis of bone. The tunnel is then filled up by circular rings of lamellae surrounding the vascular canal, both of them forming together the so called haversian system or osteon, a cylindrical motif, formed by concentric layers of lamellae and are usually oriented in the longitudinal direction of bone [16]. The micrograph in Figure 1 (B) also reveals the existence of an Haversian canal, found in the in central region of the osteon. For further details read [15].

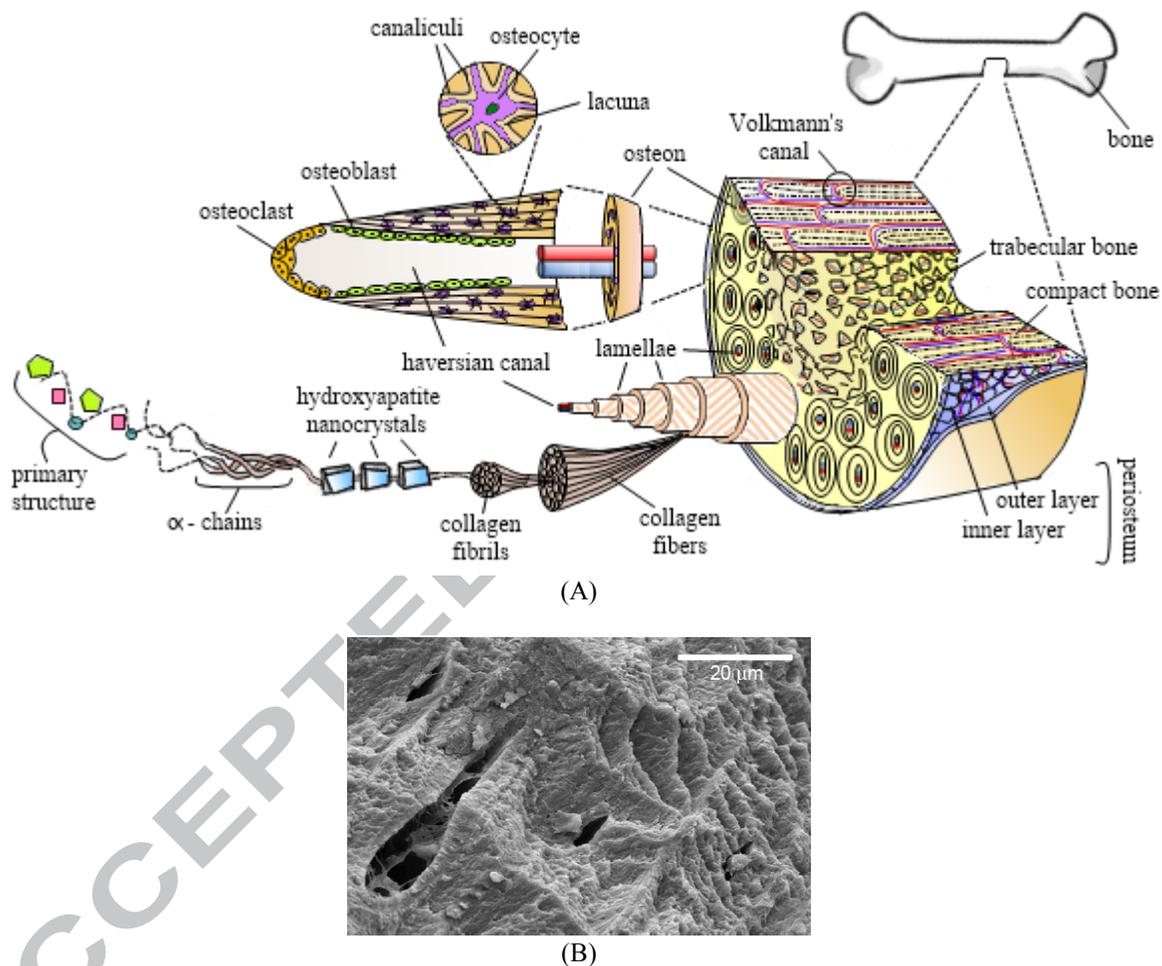


Figure 1 (A) The general structure of bone, showing its hierarchical organization; (B) Fracture surface of a bovine cortical bone revealing a lamellar morphology.

Comparing different types of bone, it can be concluded that the main determinant of mechanical properties is the amount of mineral in the tissue [17]. A clear trend between stiffness and mineral content is found when various types of bone are compared. The stiffness and strength values in the cancellous bone, varies depending on the weight or non-weight-bearing regions. Stiffness has modulus values in the range of 1GPa to 9,7GPa. Weight-bearing trabecular systems can sustain superior-inferior compression levels of as

much as 310MPa and those from non-weight-bearing regions typically fail at stresses of from 120MPa to 150MPa [18]. The near-linear relationship found in this trend shows that the failure of bone in bending is determined by the strain to which it is subjected [17]. Concomitantly, the increase in the Young's modulus is associated with a decrease in the strain at failure. Despite the importance of the mineral content, the organic matrix plays a major role in the mechanical performance of bone, contributing for its plastic deformation, and the overall hierarchical microstructure is essential for its toughness [19]. Experiments on bone fracture showed that crack propagation is prevented by various complex ways, discussed by Nalla et al. [20]. In that study it was possible to experimentally demonstrate that local criterion for fracture in human cortical bone is consistent with a strain-based criterion, rather than being stress-controlled. More correlations between mechanical properties and structure found not only in bone but in a vast number of natural materials was excellently reviewed by Meyers et al. [21].

2.3. Teeth

Mammalian tooth is a structural and functional gradient composite consisting of a hard, inert, component, the enamel, supported by the less mineralized, more resilient, and vital hard connective tissue, dentin, which is formed from and supported by the dental pulp (see Figure 2 (A)). Tooth is an engineering tool performing daily functions of mastication: teeth are subjected to stresses of about 20 MPa, 3000 times a day but their fracture is rare. It is hypothesized that this is partly due to the hardness and stiffness of enamel and partly to the toughness and relative compliance of dentin [2].

Enamel is the most highly mineralized tissue found in the vertebrate body and also the hardest, being constituted by approximately 97% mineral (w/w), 1% organic material and 2% water. The basic structure is the nanosized fibril-like carbonate apatite crystals, much larger than the apatite crystals found in bone: in newborn mammals, they can be at least 100 μm long and 50 nm diameter [2]. The crystals are bound together, forming rods, or prisms, which are in turn arranged in a decussating, plywood-like structure [17]. Figure 2 (B) shows a SEM picture of a fracture surface of enamel of a six years old infant tooth. The topography reveals the formation of unique structures consisting of aligned prisms or rods with $\sim 5 \mu\text{m}$ diameter, which run approximately perpendicular from the dentin–enamel junction towards the tooth surface. Each rod consists of tightly packed carbonated hydroxyapatite crystals, with very high aspect ratio. More details in the microstructure, detected, for example using atomic force microscopy can be found elsewhere [22]. In that work it was found, for nano-indentation experiments, that the stiffness is different in the two main direction of the rods orientation. However, Figure 2 (B) shows that, at a micron-level, different fiber orientations exist that could allow the enhancement of the isotropic behavior of the material, as discussed previously for other mineralized tissues.

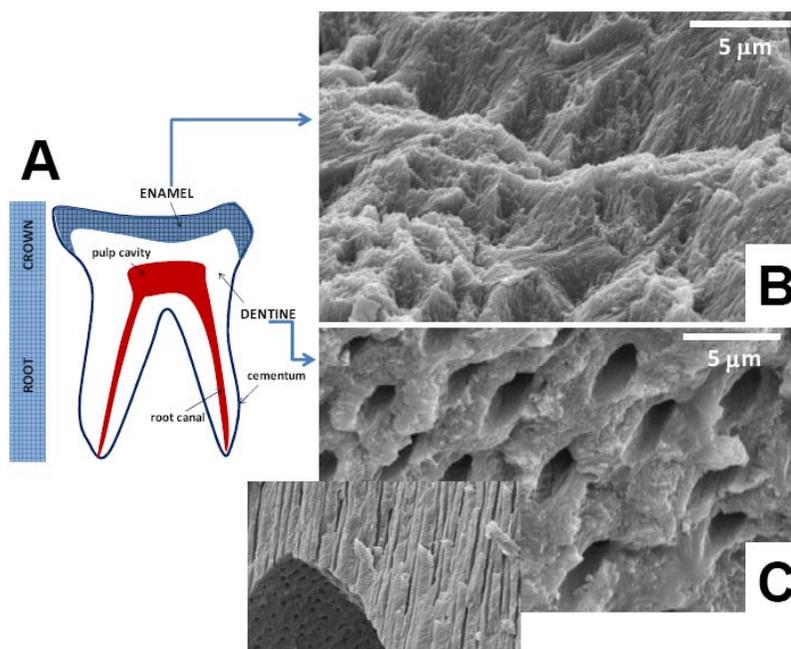


Figure 2 Mineralized structure of tooth. (A) Schematic drawing showing the enamel and dentin regions; (B) SEM micrograph of enamel and (C) SEM micrograph of dentin, showing the tubular morphology, surrounded by dense peritubular dentin – the longitudinal cut along the tubules is seen in the inset image.

Dentin has a similar composition to that of bone, its crystals being much thinner than those in enamel (ca. $2 \times 50 \times 25$ nm) [2]. The tissue comprises a network formed by randomly intertwined mineralized collagen fibrils. Dentin is permeated by tubules, as shown in Figure 2 (C) that radiate from the pulp cavity towards the dentin-enamel junction. The mechanical performance in the region of such junction was analyzed by Imbeni *et al.* in order to understand the mechanism that provides a crack-arrest barrier for flaw formed in the brittle enamel [23]: it was suggested that the interface never debonds but rather the crack penetrates and stop after a short travel through dentin.

2.4. Mollusc Shells and Nacre

Shells mainly consist of calcium carbonate (calcite or aragonite) forming multilayered microstructures, and a small amount of organic component (1 to 5 wt%), mainly located within the inter-crystalline boundaries. Despite this composition, and owing to the special composite microstructure, mollusc shells present an enhancement in toughness by three orders of magnitude with respect to non-biogenic calcium carbonate [24], [25]. In fact, the arrangement of the crystals, forces the deflection of a crack to a direction with an unfavorable stress state and prolong the crack propagation, enhancing the energy absorption in the direction of crack travel [26]. However, as it will be seen later, other mechanisms should be taken into account to explain the amazing mechanical features of mollusc shells.

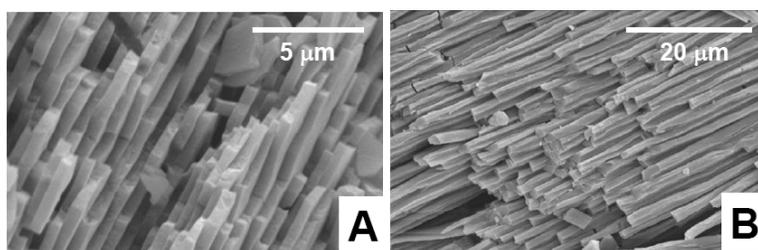


Figure 3 SEM micrograph showing the fracture surface of the nacre (A) and prismatic (B) structure of mussel shell.

In some organisms the two calcium carbonate polymorphs may be found, but separated in different layers [7], [27]. A typical example is the shell of mussel (*Mytilus sp.*), where two layers can be found: 1) an inner nacreous layer, a ‘brick-and-mortar’ of plate-like aragonite crystals (Figure 3(A), and (B)) an outer prismatic layer, with a honeycomb-like network structure of a polygonal prism formed by large crystals of calcite (Figure 3 (B)). During the growth of shells, the prismatic layer is first deposited and the nacre is added as the shell thickness increases with time [8]. These animal’s shells have both calcite (90%) and aragonite (10%). The prismatic layer is based on long calcite crystals (rhombohedral) that are produced in a space between a closely packed sheet of epithelial cells and a highly insoluble protein layer called the periostracum (covering the external surface of the shell). The nacreous layer is based on aragonite crystals (orthorhombic) deposited after the calcite layer so the mineralization is confined to the space between the cell layer and the growth front of the mineralization structure [8]. The crystallographic texture of aragonite is characterised by a nearly perfect c-axis alignment normal to the plane of the tiles. As in the prismatic layer, nucleation of aragonite tiles involves the induction of a single crystal in each nucleation site, with the particularity that in this case the orientation of the crystal is controlled in all three-dimensions relative to the structure of the organic matrix substrate [7]. Nucleation of the aragonite crystals takes place at a specific site on the surface which is known to have unique calcium-binding properties and to be rich in sulphur, presumably in the form of sulphate [7].

The nacreous layer is a laminate, with a thickness of about 0.5 μm , consisting of aragonite polygonal tablets between thin sheets of organic matrix. This matrix is formed by a protein-polysaccharide and limits the thickness of the crystals and is structurally important in the mechanical design of the shell [7], [8]. It reduces the number of voids in the wall, inhibiting crack propagation by dissipating energy related to an expanding defect along the organic layers rather than through the inorganic crystals. The organization and composition of the organic matrix in nacreous system was very well described by Addadi and Weiner [7], and reviewed later by the same group [28]. The mineral component is formed within an organic matrix composed of by β -chitin, silk-like proteins, and acidic glycoproteins rich in aspartic acid. It was hypothesised that the beta-chitin would be organised in aligned fibrils in the interlamellar sheets of the organic matrix, whereas the silk would be placed within the sheets [28]. Nacre is stiff ($E=60\text{--}80$ GPa) while maintaining a relatively high toughness ($J_{IC}=1.5$ kJ/m², this is about 1,000 times the toughness of aragonite) [29]. Other mechanisms have been also proposed to explain the remarkable mechanical performance of nacre. A strength anisotropy perpendicular to the layers of 5 MPa vs. 540 MPa [21, 25, 30] and a relatively small difference in tensile and compressive strength of 170 MPa vs. 230 MPa [31], justifies the high toughness, possibly attributed to the existence of intertile

mineral bridges in combination to the organic “glue”. Figure 4 summarizes the strength of nacre with respect to various loading directions.

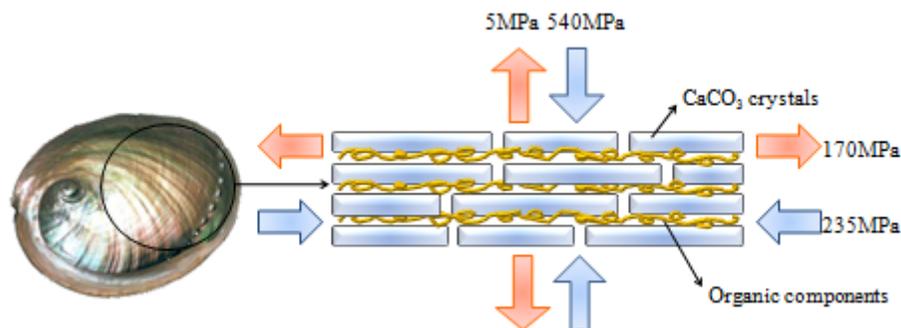


Figure 4 Compressive and ultimate tensile strengths of nacre under different loading direction.

Wang et al. examined the inelastic deformation of nacre, and conclude that this would involve interlamellae shearing [32]; moreover, these authors reported the existence of nanoasperities onto the aragonite tablets, that could provide resistance to interfacial sliding. Based on atomic force microscopy (AFM) and scanning ion conductance, Schaffer et al. [33] suggested that mineral bridges could exist between the successive aragonite tablets, which were confirmed by Song et al., using TEM [34]. This particular nanostructure significantly influences the mechanical performance of the organic matrix layers of nacre, helping to arrest crack propagation [35]. Moreover, it was found that the individual aragonite platelets in nacre consist of the assembly of cobble-like polygonal nanosized grains (with sizes of about 32 nm) providing a ductile nature to such microstructures [33]. Such deformability of the aragonite platelets is relevant for the nacre's fracture toughness. A more recent work reports in-situ AFM observations of the nanogranular texture of the aragonite platelets during mechanical deformation [36]. Under this process nanograin rotation and deformation occur, facilitated by the existence of the biopolymer spacing between the nanograins, which will contribute to energy dissipation in nacre. The water present at the nanograin interfaces also contributes for the viscoelastic features in nacre. In fact, it was found that water has a significant positive effect on the macroscopic mechanical properties of mollusc shells [37], [38].

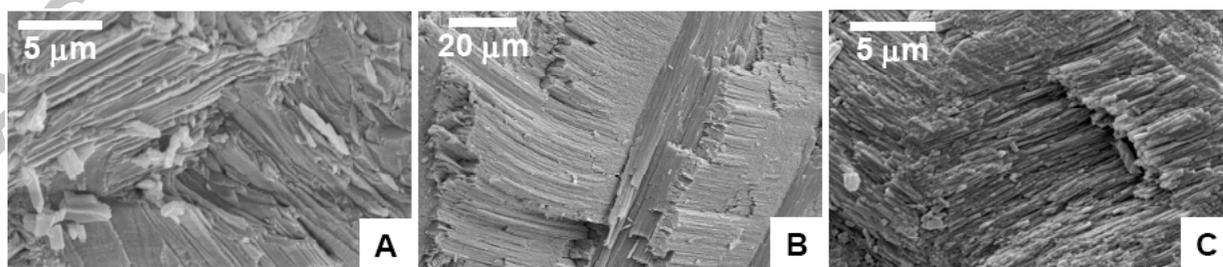


Figure 5 Fracture surface of the cross-lamellar structure of an oyster shell (A); a conch shell (*Muricopsis sp.*) (B) and a snail shell (*Helix sp.*) (C).

Other morphologies, different from the prismatic and nacreous ones can be found in seashells. Figure 5A shows the typical crystalline arrangement in an oyster shell, where it is clear a non-uniform arrangement of the elongated crystals. We have then an additional upper level in the hierarchical organization of the shell in a cross-lamellar like organization, which will have a role in crack deflection during the fracture process. Similar microstructures were analysed in another seashell [39], [40]. The cross-lamellar structure is a three-dimensional array of closely packed aragonite crystals, which are packed together tightly with their long axes and straight sides all aligned to form a sheet or lamella tens of microns wide and thick [41]. As previously mentioned in Section 2.1., this kind of morphology will enhance the isotropy of the macroscopic material.

Cross-lamellar morphologies can also be detected in gastropods. The shells of a conch (*Muricopsis sp*) and of a terrestrial snail (*Helix sp.*) observed in the present work exhibit principally a cross-lamellar structure – see Figures 5 (B) and 5 (C), respectively.

The micro-architecture of nacre (mother of pearl) has been classically illustrated as a ‘brick-and-mortar’ arrangement. It is clear now that hierarchical organization and other structural features play an important role in the amazing mechanical properties of this natural nanocomposite. The more important structural characteristics and mechanical properties of nacre are exposed as a base that has inspired scientists and engineers to develop biomimetic strategies that could be useful in areas such as materials science, biomaterials development and nanotechnology [42].

3. The importance of the interface in biogenic composites

Although presenting a hierarchical organization, any biocomposite is a complete structural system in itself, composed by different levels that are held together by specific interactions between the components. Therefore, the final mechanical and functional performance will be highly influence by the surface-to-surface interactions, including the interactions between the organic and inorganic components. Whatever the nature of the bonding between levels, adequate adhesion is required for system structural integrity. For example, it was suggested that one of the mechanisms associated with the good toughness of bone is that cracks propagation is prevented by unbroken collagen fibrils that bridges the crack [20]. In another study the total deformation of bone was studied together with the local deformation of the fibrils and mineral particles, by combining *in situ* tensile testing with high brilliance synchrotron X-ray diffraction and scattering [43]. The results were consistent with a hierarchical and coupled deformation mechanism starting from the nanoscale, where the organic matrix is relevant in the amount of strain transferred to the mineral platelets. This is a clear indication of the importance of the organic component, and its good liaison with the apatitic phase, on the mechanical performance of a mineralized tissue. The importance of the nano microstructure of the inorganic component in nacre for its mechanical properties has been referred to in section 2.4. The particular interactions existing between the organic and aragonitic components also play an important role in the toughness of nacre. The inorganic insoluble layer between the nacre tablets can be seen as

an adhesive that holds the tablets together. Smith et al. used AFM to perform force-extension experiments in single-molecules of the organic material that was exposed on a freshly cleaved nacre surface [44]. The curves obtained did not exhibit a smooth and continuous shape as usually found in soft materials; instead, the individual fibers elongate in a stepwise manner, producing a series of sawtooth jumps, as folded domains or loops are pulled open. Such “sacrificial” (either intra- or interchain) bonds existing within the structure of the organic molecules are believed to be in the origin of such peculiar behavior found on the nano-mechanical features of such macromolecules and will provide high energy of break.

The same kind of sacrificial bond and hidden length mechanism contributes to the mechanical properties of the bone composite [45]. In this case it was suggested that bone consists of mineralized collagen fibrils that are glued together by a non-fibrillar organic matrix. AFM measurements were also performed to get nano-mechanics information, where two pieces of bone are put in contact in solution, one on the AFM cantilever and one as a sample; the pieces were pressed together and pulled apart during which the forces were measured. It was seen that when the glue between the two pieces is stretched, energy is dissipated through rupturing of sacrificial bonds and the stretching of hidden length. This mechanism will contribute to the toughness of bone by increasing the amount of energy necessary for a crack to propagate. It was also suggested that specific interactions between the collagen-rich organic matrix and the ceramic nanoparticles could be on the origin of the minimum in the loss factor observed at 37°C when one performs viscoelastic measurements as a function of temperature [4]. Such finding could be assigned to the fact that the molecular motions associated to damping may damage the bone structure. The minimization of damping at meaningful temperatures and frequencies may then reduce this harmful process.

4. Biomimetic calcium phosphate coatings in the biomedical area

One of the most important applications that can arise from the study of biomineralization is in the treatment of medical pathologies or injuries in calcified tissues. In orthopedic applications the surface properties of implants play a fundamental role for the fixation to the bone tissue, in order to assure their long term function. However, artificial materials implanted into bone defects are usually encapsulated by a fibrous tissue, isolating it to the surrounding bone and thus compromising their use in bone repair. This tendency has been overcome by using or coating the implant with bioactive ceramics or glasses that spontaneously integrate with bone *in vivo* [41]. Among these materials, Bioglass®, BG, in the Na₂O-CaO-SiO₂-P₂O₅ system, was found to exhibit excellent bone bonding capability and has been used clinically since 1985 [46]. Upon implantation, bioactive ceramics and glasses produce a layer of apatite at the interface with bone, consisting of nano-crystals of carbonate-ion-containing apatite that has a defective structure and low crystallinity (see [41] and refs. cited therein). Much work has been developed to enhance the bioactivity of materials. They may involve procedures such as the introduction of bioactive particles of

ceramics or glasses into the polymer (bioactive composites) or over its surface, or by chemically modifying the surface of the polymeric material, with groups that enhance the nucleation and growth of apatite. The so-called biomimetic preparation of calcium phosphate coatings on implant materials has emerged as a new concept and several methodologies have been proposed, especially implemented in polymeric-based systems, as it can be carried out at low temperatures [47], [48].

An essential question is to assess the bioactive performance of materials *in vitro*. It was proposed that the formation of bonelike apatite *in vivo* could be reproduced in a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma. Therefore, *in vivo* bone bioactivity of a material can be predicted from the apatite formation on its surface upon immersion in SBF [49-51].

An example is given in Figure 6 where one can see the formation of a calcium phosphate layer in constructs for bone tissue engineering applications. Such kind of biodegradable porous structures can be seeded with the patient's own cells and differentiated into osteoblasts; when enough tissue is formed *in vitro* within the material, the hybrid construct is implanted in the bone defect in order to promote the formation of new bone. In a particular study PLLA/Bioglass® were prepared by compression molding followed by salt leaching. This allows for the production of porous structures with smooth surfaces (see Figure 6A-left). Upon immersion in SBF an apatite layer is formed exhibiting a typical cauliflower morphology, which is composed by nano-sized carbonated hydroxyapatite crystals, i.e. very similar to the ceramic component found in bones (Figure 6A-right). The combination of salt and another polymeric water soluble porogen, such as poly(ethylene oxide), PEO, permits the formation of pores with a textured surface due to the fingerprint left by PEO spherulites that are leached out during the scaffold processing [52] – see Figure 6 (B) - left. It was found that when Bioglass® is present in such textured scaffolds the ceramic formed upon immersion in SBF has a completely different nature that could lead to a different biological performance of the implant – see Figure 6 (B) - right [53]. This result demonstrates the relevance of surface topography in the morphology of the calcium phosphate coating that is developed during immersion in SBF.

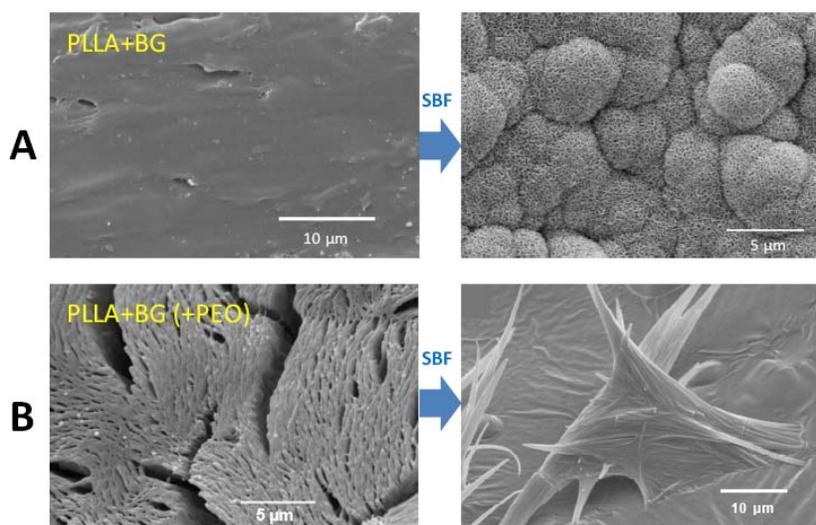


Figure 6 (A) Representative SEM image showing (left) the smooth surface of a porous PLLA/Bioglass® scaffold and (right) after one day of immersion in SBF, where the typical cauliflower-like morphology of the apatite layer is observed. (B) Representative SEM image showing (left) the rough patterned surface of a porous PLLA/Bioglass® scaffold where also PEO was used as a porogen and (right) after one day of immersion in SBF, where a completely different calcium phosphate coating was formed. Data based on results from [53].

Calcium phosphate coatings can also be used as reservoirs for the release of relevant therapeutic agents. An interesting concept is the inclusion of cytokines or morphogenetic factors, such as BMP-2, into biomimetic calcium phosphate coatings [54], which enhance the performance of the scaffold.

There is a strong interest in developing “smart” materials for a range of biomedical applications, which are able to respond to the trigger of different external variables, such as temperature, pH or ionic strength [55]. Such devices can be used in controlled drug delivery, cell culture substrates or sensors/actuators. In this context it would be interesting if one could trigger the onset of mineralization onto the surface of a biomaterial when immersed in SBF upon some change of an external variable, such as temperature, pH, light or salt concentration. For example, “smart” surfaces were produced in poly(L-lactic acid)/Bioglass® composites where a temperature-responsive polymer was grafted onto the surface: the precipitation of bone-like apatite occurred at physiological temperature, but was prevented at room temperature [56]. Such concept was extended to pH-responsive systems, where chitosan was grafted onto such composites [57]: in this case calcification occurred just at physiological pH and was prevented in acidic media, where the surface is more hydrophilic. Calcified responsive particles were also prepared where the release profile of a previously encapsulated drug could be dependent on both temperature and pH [58].

Mimicking biomineralization under microgravity was performed by Sinha *et al.* [59]. The authors carried out on-board during the flight of first recoverable space capsule (SRE-1) launched by Indian Space Research Organization, a polymer matrix mediated synthesis of

hydroxyapatite nanoparticles under microgravity. In contrast, of a defect free large crystal formation under microgravity in solution crystallization, the present study revealed an order of magnitude reduction in the dimensions of hydroxyapatite nanoparticles synthesized by a biomimetic process with respect to its 1 g counterpart.

5. Biomimetic nacre-inspired nano/micro laminated materials and biomaterials

Biomimetic strategies should not attempt to copy directly the structures or functions of biological composites but rather gather key concepts from these systems that can be somewhat adapted within a synthetic concept. Therefore, as commented by Green *et al.*, biomimetic composites should be habitually less complex than their biological counterparts and, to date, hierarchical architectures as observed in biological composites, remain outside the current technologies [60]. Advances in polymer technology have permitted to produce hierarchical structures in plastic pieces, using, for example, semi-crystalline or liquid-crystalline polymers [61].

Much effort has been made to mimic the general structure and properties of natural composites, especially the synthetic production of nacre-like materials. Here we present the latest advances on the synthetic design and production of nacre-inspired materials, in particular to be used in biomedical applications. The basic structural motif in nacre is the assembly of oriented plate-like aragonite crystals with a 'brick' (CaCO₃ crystals) and 'mortar' (macromolecular component) organization. Many works recognized that such structure would be associated with the excellent mechanical properties of nacre, and biomimetic strategies have been proposed to produce new layered nanocomposites [42].

A simple example of nacre mimicking was the production of laminated a Si₃N₄/BN composite, using a roll compaction technique, imitating the layered microstructure found in nacre [62]. The fracture surface of this material exhibits clear crack deflection and the fracture toughness was 28 MPa m^{1/2} and works of fracture more than 4000 J/m². It would be much more interesting to fabricate nanolaminate structures at mild conditions, i.e. aqueous solutions and environmental temperatures and pressures. Moreover, it would be also important to have a good control of the organic/inorganic interface. Manne and Aksay reviewed some methodologies of producing nanolaminates [63], based on the use of inorganic particules. Four categories are addressed and discussed, namely Langmuir-Blodgett deposition, covalent self-assembly, alternating sequential adsorption and intercalation of organics into layered inorganic structures. Another approach proposed by Sellinger *et al.* was based on a self-assembly process [64]. The process starts with a solution of silicates, coupling agents, surfactant, organic monomers and initiators in a water/ethanol mixture; during dip-coating micellar structures are formed and assemble into interfacially organized liquid-crystalline mesophases, thereby simultaneously organizing both the inorganic and organic precursors into the desired laminated structure. Organic polymerization, combined with continuous inorganic polymerization, lock-in the nanocomposite morphology through covalent bonds within the organic-inorganic interface. Note that most of the approaches presented do not enable the production of thick

nanocomposites with pre-defined complex geometries. Thick hybrid films, based on nanocomposites containing clay with layered structure were produced by Bonderer et al., exhibiting excellent stiffness and strength [65]. Nanostructured organic-inorganic films were produced by Tang et al., also based on the layer-by-layer method, in which layers with more than 5 μm could be obtained [66]. The multilayered films were produced by sequential immersion of a glass slide in solutions of a polycation and anionic montmorillonite clay. The tensile strength of the prepared multilayers approached that of nacre, whereas their ultimate Young modulus was similar to that of lamellar bone. In another work the layer-by-layer technique was combined with chemical bath deposition to prepare TiO_2 /organic polymer multilayered films with a nacre-like architecture [67]. Osteoconductive glass-ceramic nanoparticles were produced by a sol-gel methodology and showed to be bioactive when combined with polymers [68], [69]. Layer-by-layer nanostructured hybrid coatings were also obtained by the sequential deposition of a substrate in a suspension of such bioactive glass-ceramic nanoparticles (exhibiting a negatively charged surface) and a solution of a positively charged polymer (chitosan) [70] – see Figure 7. Such biodegradable coatings were found to promote the deposition of apatite upon immersion in SBF (Figure 7) and are believed to have potential to be used in a series of orthopedic applications.

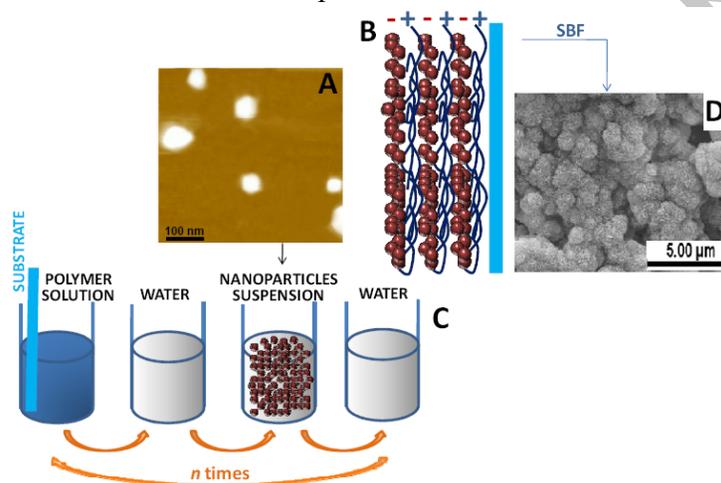


Figure 7 Bioactive nanoparticles (AFM image in A) were used to produce multilayered coatings (B) by alternate dipping a substrate in a chitosan solution, water and nanoparticles suspension (C). Upon immersion in SBF an apatite layer could be detected onto the surface (D). Data based on results from [70].

During the formation of sea ice the solutes present in sea water are expelled from the forming ice and entrapped within channels between the ice crystals; such principle was applied by Deville et al. to ceramic particles dispersed in water to build nacre-like architectures [71]. First, layered materials are prepared through a freeze-casting method; the porous scaffolds are then filled with a second phase, e.g. an organic component, in order to produce a dense composite. Such simple methods should allow the production of layered composites with complex shapes and very interesting mechanical properties.

We referred before to the fact that the formation of nacre involves the use of organic macromolecules as templates for the nucleation of the minerals and the control of the final material's shape. It was shown that a nacre-mimetic architecture could be synthetically reproduced through an appropriate combination of inorganic crystals and organic polymers

[72], [73]. The specific interactions existing between the two components during crystallization of K_2SO_4 in the presence of poly(acrylic acid) generate the nanoscopic architecture (20 nm diameter blocks), and the switching between the modes of growth explains the formation of the macroscopic structure (microscopic nacre-like layered structure) [72]. Similar experiments were carried out with $CaCO_3$ and poly(acrylic acid), where it was seen that, as in real biominerals, the synthetic mineral was generated from bridged nanocrystals with incorporated organic polymer [73]. Such bottom-up approaches may be useful to the design and synthesis of biomaterials for hard-tissues, such as scaffolds for bone replacement or regeneration [74].

Other bottom-up approaches are based on self-assembling. Also inspired by this natural material, Launey *et al* demonstrated that the concept of hierarchical design can be applied to conventional compounds such as alumina and poly(methyl methacrylate) (PMMA) by using ice-templated structures. With a flexible approach (that can be extended to other materials) they produced bulk hybrid materials with exceptional toughness that can be nearly 300 times higher (in energy terms) than either of their constituents. Like in nacre, the best synthetic materials reflect the natural concept of a hard ceramic phase providing for material strength, separated by a softer “lubricant” phase to relieve stresses in order to enhance toughness. The result is a high toughness ceramic, with strength of 200 MPa and a fracture toughness that is ≈ 300 times (in J-terms) larger than the main constituent, Al_2O_3 , and exceeds values of $30 \text{ MPa m}^{1/2}$ ($J_c \approx 8000 \text{ J m}^{-2}$). Future work is being directed towards the formation of hybrid materials with much higher inorganic contents, the manipulation of the properties of the soft lubricating phase and extending this concept to other material combinations. In particular, the authors are attempting to process ceramic scaffolds infiltrated with higher melting point metals with the objective of developing strong and tough ceramic-based materials that can operate at elevated temperatures [75]. T.-H. Lin *et al* reported a simple electrodeposition technology that enabled the creation of an inorganic-organic nanocomposites assembling gibbsite ($Al(OH)_3$) nanoplatelets/polyvinyl alcohol in a single step. The oriented layered nanostructures mimic the ordered brick-and-mortar nanostructure found in the nacreous layer of mollusk shells. The electrodeposited inorganic/organic nanocomposite films are obtained by applying direct-current electric field that enables the preferential alignment of gibbsite nanoplatelets and the co-deposition of non-ionic-type polymer between the inorganic nanosheets. The resulting self-standing nanocomposite films are optically transparent and flexible, even though the weight fraction of the brittle inorganic phase is higher than 80% [76].

Tseng *et al.*, fabricated a nacrelike ZnO structure with three levels of hierarchy by a biomimetic method under mild conditions where the biopolymer gelatin was used as structure directing agent. The gelatine contains many polar amino acids, as surface protecting agent for the polar surfaces of ZnO. They found that the ZnO-gelatin microcrystal with well-defined hexagonal twin plate shape is built by the stacking of nanoplates. The irregularly edged nanoplates can adjust themselves to each other throughout the microcrystal, resulting in a roughly hexagonal edge. Similar to nacreous

architecture, the nanoplate of ZnO was constructed from the oriented attachment of ZnO nanoparticles. The synthesis of the hierarchical ZnO structure in this paper could lead to new approaches to control the crystal size, orientation, and spatial patterning [77].

Ekiz *et al.* used a novel technique called Hot-press Assisted Slip Casting (HASC) to produce artificial nacre-like laminar composites. This method combines hot-pressing and slip-casting to improve alignment and volume fraction of the reinforcement, allowing the production of Bulk nano-laminar composites. Alumina flakes were used as filler in an epoxy matrix. Flexural tests on Chevron notched specimens revealed a high work-of-fracture in the case of the fabricated composites reaching to 254 J/m². Main fracture mechanism is debonding of flakes from the matrix. With its high volume fraction (60%) of reinforcement phase and high degree of flake alignment, a nacre-like microstructure was achieved with a relatively efficient, cost effective and simple hybrid conventional method. The authors believe that with suitable raw materials HASC can be a very promising means to fabricate bulk biomimetic high work of fracture nano-laminar composites that can be used in numerous applications, increasing volume fraction of the filler and decreasing porosity that can lead to maximized mechanical performance.

Cai and Tang [78] suggested a new model of “bricks and mortar” based on the biological aggregation of apatite nanoparticles. An inorganic phase, amorphous calcium phosphate, acts as “mortar” to cement the crystallized “bricks” of nano-hydroxyapatite. Meanwhile, biological molecules control the nano-construction. By using hydroxyapatite nanospheres as the building blocks, highly ordered enamel like and bone-like apatite are hierarchically constructed in the presence of glycine (Gly) and glutamate (Glu), respectively.

6. Tissue engineering of mineralized structures

The overall goal of tissue engineering is to create functional tissue grafts that can regenerate or replace defective or worn out tissues and organs [79]. Tissue engineering strategies combines cells, biodegradable scaffolds, and bioactive molecules to recapitulate natural processes of tissue regeneration and development. The scaffold is basically an artificially substrate that will support cell proliferation and differentiation while maintaining its stability. Along the development of new tissue, the scaffold must degrade. These processed materials are viable under *in vitro* and *in vivo* conditions, allowing the integration of the scaffold with the living tissues [80]. Material choices are guided by the need to restore cell signalling and to match the mechanical behaviour of the tissue being engineered [81]. An ideal scaffold need to accomplish certain requirements to be able to stimulate the body’s repair mechanisms. These include having a pore network large and open enough for cells and blood vessels to penetrate and the ability to bond to bone. [82] In the orthopedic area, the ideal scaffold should promote early mineralization and support new bone formation while at the same time allowing for replacement by new bone. Osteoconductive scaffolds may be readily prepared by including bioactive ceramic or glasses within the polymeric matrix [83].

When studying biomineralization processes, calcium phosphates are of special importance because they are the most important inorganic constituents of biological hard tissues in vertebrates. They consist of the same ions as the mineral in natural bones [84]. Biologically formed calcium phosphates are often nanocrystals that are precipitated under mild conditions. They are disposed in complicated hierarchical structures, always based in small blocks of nanometer size scale. Mimicking the formation of natural calcium phosphate hard tissues contributes significantly to the biological function of engineered materials. Many advances have been made in biomaterials with the rapid growth of nanotechnology, allowing the possibility of applying nano-calcium phosphates three-dimensional scaffolds in the repair of hard and soft skeletal tissues [80], [85],[86].

Cai and Tang [78] present an interesting reflection over nano-calcium phosphates, showing that the capability of synthesizing and processing nano-calcium phosphates with controlled structures and topographies, in an attempt to simulate the basic nano-units of natural materials, provides the possibility of designing novel proactive bioceramics necessary for enhanced repair efficacy. Specifically, nano hydroxyapatite can be seen as an ideal biomaterial able to mimic the building units of biological tissues such as bone, dentin, and enamel being of an extreme importance in hard tissue engineering due to its good biocompatibility and bone/enamel integration. The various primary positive results, regarding the biocompatibility and biomimicry of novel nanostructured bioceramics to natural bone, merit further confirmation (as a deep study of how these materials interact with cells) and also express a concern with the biological security of using nano particles, due to the danger of reaching the circulatory system by penetration into blood vessels. As bone biogenesis is thought to occur by templated mineralization of hard apatite crystals by an elastic protein scaffold, Song *et al* [74] attempted to recapitulate this process with synthetic biomimetic hydrogels (cross-linked polymethacrylamide and polymethacrylate polymers) functionalized to mimic the mineral nucleating proteins of bone. Strong adhesion between the organic and inorganic materials was achieved for hydrogels functionalized with either carboxylate or hydroxy ligands. They investigated the integration of biomimetic mineral-nucleating ligands with calcium phosphates using a mineralization approach described elsewhere [87], discovering that the morphology and crystallinity of the mineral, as well as the binding strength at the polymer-mineral interface, were governed by the structure and density of the templating ligands. These results provide a framework for generating synthetic composites with defined organic/inorganic interfaces similar to natural bone.

The current challenge in bone tissue engineering is to fabricate a bioartificial bone graft mimicking the extracellular matrix (ECM) with effective bone mineralization, resulting in the regeneration of fractured or diseased bones [88]. In order to evaluate the potential of using nanostructured substrates for bone tissue regeneration, Prabhakaran *et al* [88] used electrospinning to prepare biocomposite polymeric nanofibers blending poly-L-lactide (PLLA), collagen and nano-hydroxyapatite. Osteoblasts grown on PLLA/collagen/hydroxyapatite nanofibrous scaffolds showed higher cell proliferation, and

increased ALP activity and mineralization, than the PLLA and PLLA/hydroxyapatite scaffolds. Hence the obtained biocomposite PLLA/collagen/hydroxyapatite nanofibers revealed to be promising structural scaffolds with suitable cell recognition sites, biocompatibility, osteoconductivity and sufficient mechanical strength for bone tissue engineering. Also based on this principle, nano-hydroxyapatite/collagen based composites, inspired from research on natural bone, have received great attention. The composites are prepared by directly mixing the nano-hydroxyapatite and collagen. Nano-hydroxyapatite is produced using modern ceramic technologies, while collagen is purified from animal tissue as fixing agent for hydroxyapatite. The weak binding between hydroxyapatite and collagen make them no cooperation effect in vivo for bone defects repair. The collagen degrades fast, but hydroxyapatite ceramic remains in the original form which do not attend the remodeling progress of bone. Researchers have also tried to develop mimetic methods to prepare nano-hydroxyapatite/collagen composites. One method involves the immersion in a simulated body fluid (SBF), used to improve the biocompatibility of conventional metal, alloy and polymer implants. Another method involves co-precipitation of collagen fibrils and nano-hydroxyapatite spontaneously, and is a promising route for achieving the same hierarchical structure in synthetic materials as in bone [89]. Cui *et al* [89] present a review on self-assembly of mineralized collagen composites including recent work involving biomimetic synthesis of new materials with the structure of mineralized collagen, focusing mainly on materials containing type I collagen, with mineralization by Ca-P crystals.

A new class of biomimetic molecules that can form gels from aqueous solutions was proposed by Stupp and co-workers [90], [91]. They introduced the concept of amphiphilic peptide, consisting of a peptide sequence covalently bonded to a very hydrophobic segment that is stable and neutral in aqueous solution. By changing the pH or the electrolyte environment (e.g. put the molecules in contact with physiological fluids) the molecules self-assemble giving rise to a complex 3D network of nanofibers and to mechanically consistent gels with very high water contents, being adequate to act as a synthetic extracellular matrix to be used in different applications in the field of regenerative medicine. By designing the peptides sequences appropriately it is possible to induce the precipitation of bone-like apatite onto such nanofibers [90], indicating that they could find applications in the orthopedic area.

Sol-gel derived bioactive glasses are also relevant on mineralized structures tissue engineering because they have a nanoporosity that can control degradation rate [86]. They can be foamed to produce scaffolds that mimic cancellous bone macrostructure. Bioactive glass foams with optimised nanoporosity are strong in compression; however, they have low toughness and pore strength when loaded in tension. Therefore an ideal scaffold would have all the properties of the glasses with enhanced toughness. This can only be achieved by creating new nanoscale composites. Resorbable polymers must interact with the silica based inorganic network at the nanoscale to maintain bioactivity and controlled resorption. A scaffold like this would regenerate diseased or damaged bone to its original healthy state [82].

In order to understand the complex mineralization processes involved in bone-matrix mineralization, and adapt these strategies to the design of materials, Spoerke *et al* [92] developed an artificial, *in vitro* biomineralization process that utilizes a nanofiber gel as a substrate for biomimetic hydroxyapatite mineralization in three dimensions. The system employs alkaline phosphatase (a natural enzyme secreted by osteoblasts, which liberates phosphates necessary for HA mineralization from organic phosphates) and a phosphorylated, anionic nanofiber gel matrix to template HA nanocrystals with size, shape, and crystallographic orientation resembling natural bone mineral. Although the authors don't intend to mimic, functionally or structurally, any single bone protein, they believe that the assembly of peptide amphiphile nanofibers into a scaffolding framework could lead to biomimetic materials to promote bone regeneration, or the synthesis of hybrid materials with crystallographically defined structures. Data suggested that neither a nanofiber matrix alone, nor calcium enrichment alone will promote the ALP-mediated template mineralization observed in the PA gels, both spatial and temporal elements are necessary to achieve biomimetic mineralization in synthetic materials.

A high degree of hierarchy, gives bone its optimal bio-physical response. The current inorganic scaffold production processes do not allow the generation of a biomimetic organized hierarchical structure, due to the consistent limitations in the current chemical processing technology for biomaterials. The development of hierarchically organized bone scaffolds would surpass the current solutions in the synthetic bone substitutes matter.

Some authors [93-96] were inspired by Nature. Tampieri *et al* [93] was specifically inspired by natural wood templates, in the development of hierarchically structured biomaterials. Wood exhibits a remarkable combination of high strength, stiffness and toughness at low density due to the unique hierarchical architecture of the cellular micro-structure. Hence, the alternation of fibre bundles and channel-like porous areas makes wood an elective material to be used as a template in starting the preparation of a new bone substitute characterized by a biomimetic hierarchical structure. In their work, the authors developed a new biomimetic hydroxyapatite bone scaffold having highly organized micro- and macro-porosity, by implementing a multi-step procedure involving chemical-physical transformations of a natural wood template: pyrolysis of ligneous raw materials to produce carbon templates; vapour or liquid calcium infiltration to transform carbon into calcium carbide; oxidation process to yield calcium oxide; carbonation by hydrothermal autoclave treatments in controlled environment for further conversion to calcium carbonate and, finally, phosphatization through a hydrothermal process. This complex chemical process leads to the synthesis of biomimetic hydroxyapatite hierarchically organized scaffolds. Such synthesized structures maintain the 3D porous morphology of the starting native wood, thus allowing cell in-growth and reorganization and consequently providing the necessary space for vascularisation due to the unidirectional oriented pore structures on the micrometer scale. The hierarchical architecture of the wood cellular microstructure and the hydroxyapatite constituting phase allow this new biomimetic material to be considered as an innovative charming inorganic scaffold for bone regeneration and engineering.

7. Concluding remarks

Nature offers a variety of hard materials exhibiting different hierarchical and oriented structures using a limited source of minerals and organic molecules. Such systems are produced in a bottom-up fashion under mild and aqueous solution conditions. These materials are designed to fulfill their structural and functional requirements. We believe that lessons taken from the study of the structure-properties relationships of biocomposites and the mechanisms of biogenic composite formation can inspire the development of new concepts both for the design and the processing of man-made materials. The field of biomimetics has been developed faster in the last years, due to the recent developments of (i) biology applied to materials science, including the use of biomaterials in tissue engineering and regenerative medicine; (ii) nanoscience and nanotechnology and (iii) supramolecular chemistry. We believe that such tools will enable the translation the enormous amount of work that has been accumulated from the observation of Nature, namely the structure-properties/functions relationships found in natural nanocomposites, into useful devices.

Acknowledgments: This work was supported by the Portuguese Foundation for Science and Technology (FCT), through project PTDC/QUI/69263/2006.

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