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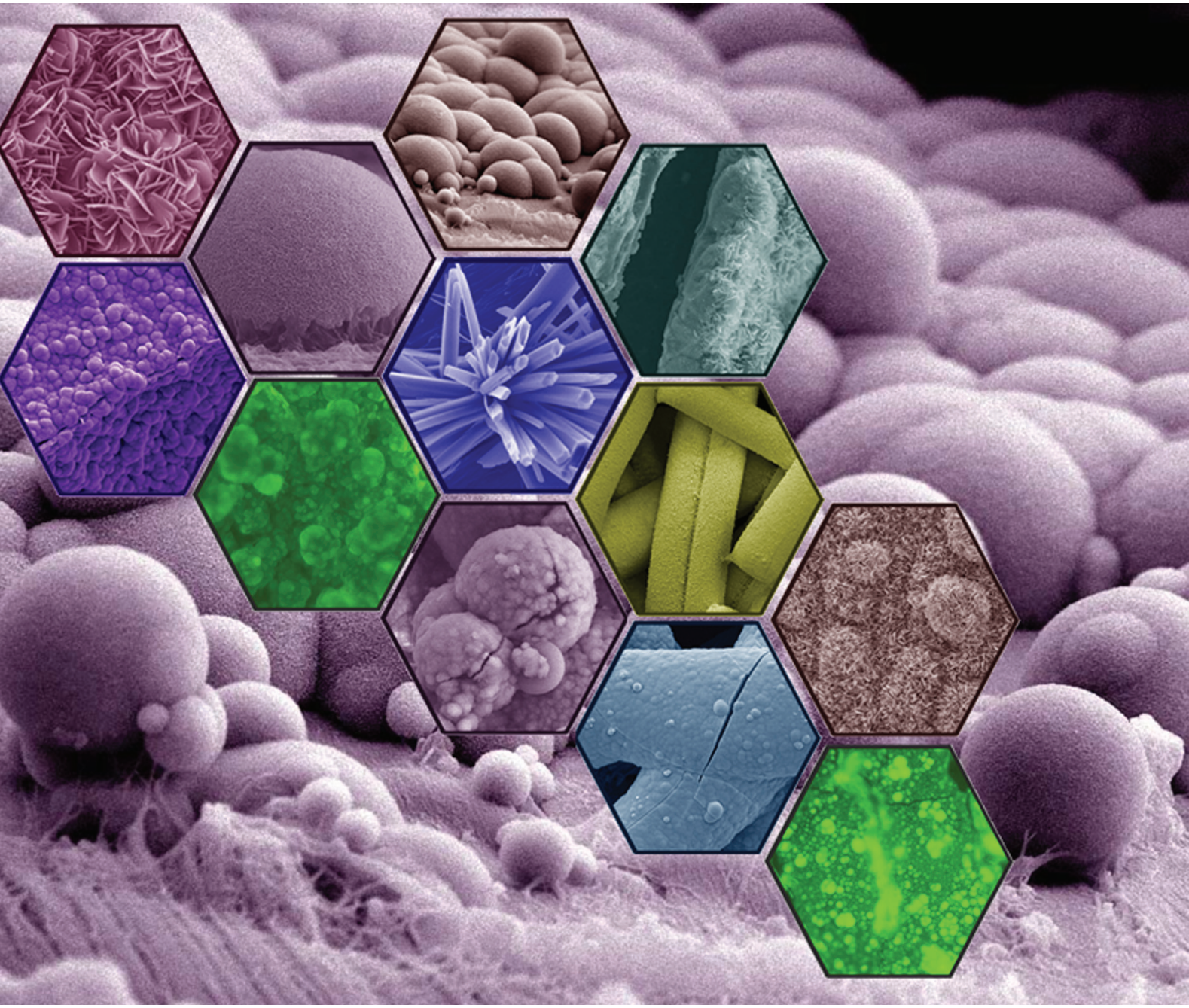

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FEATURE ARTICLE
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Designing biomaterials based on biomineralization of bone

N. M. Alves,[ab] I. B. Leonor,[ab] H. S. Azevedo,[ab] R. L. Reis[ab] and J. F. Mano*[ab]

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In nature, organisms control crystal nucleation and growth using organic interfaces as templates. Scientists, in the last decades, have tried to learn from nature how to design biomimetic biomaterials inspired by the hierarchical complex structure of bone and other natural mineralised tissues or to control the biomineralization process onto biomaterials substrates to promote the osteoconductive properties of implantable devices. The design of synthetic bone analogues, *i.e.*, with a structure and properties similar to bone, would certainly constitute a major breakthrough in bone tissue engineering. Moreover, many strategies have been proposed in the literature to develop bioactive bone-like materials, for instance using bioactive glasses. Fundamental aspects of biomineralization may be also important in order to propose new methodologies to improve calcification onto the surface of biomaterials or to develop bioactive tridimensional templates that could be used in regenerative medicine. In particular, it has been shown that some chemical groups and proteins, as well as the tridimensional matrix in which calcification would occur, play a fundamental role on the nucleation and growth of hydroxyapatite. All these distinct aspects will be reviewed and discussed in this paper.

1. Bone: a complex structure

Hard tissues in vertebrates, such as bones, are exquisite examples of structures arranged from nanometre to macroscopic scale, produced by natural biomineralization using organic templates to control the growth of the inorganic phase. Bone is a hierarchically structured composite material which has been well studied by the materials engineering community because of its unique structure and mechanical properties.[1] From a materials science perspective, the nanostructure of bone is intriguing and even quite difficult to define. Bone structure is, however, increasingly being understood as a result of better analytical and high resolution microscopy instrumentation. The fundamental subunit is mineralized collagen fibril that consists of self-assembled triple helices of collagen molecules. Hydroxyapatite nanocrystals grow on these assembled fibrils, with their crystallographic c-axes aligned with the fibril long axes. It is still not entirely understood whether the hydroxyapatite crystals are directly nucleated on the collagen fibrils, or if the hydroxyapatite

[a]*3B's Research Group - Biomaterials, Biodegradables and Biomimetics, Dept. of Polymer Engineering, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Zona Industrial da Gandra, S. Cláudio do Barco, 4806-909 Caldas das Taipas, Guimarães, Portugal. E-mail: jmano@dep.uminho.pt*
[b]*IBB- Institute for Biotechnology and Bioengineering, Braga, Portugal*

N. M. Alves

Natália M. Alves received her PhD in Science and Engineering of Polymers in 2004 from the University of Minho, and currently is an Assistant Professor at the same university. Her research activity, developed at the 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, is essentially focused on smart materials for biomedical applications (drug delivery and tissue engineering) and thermal and physical properties of biomaterials. She is author of 36 papers in international journals, 42 communications presented in International/National conferences and 3 book chapters. She has been involved in distinct research projects and in the orientation of several students.

I. B. Leonor

Isabel B. Leonor received her PhD in Materials Sciencel Technology-Biomaterials from the University of Minho (UM), under the supervision of Prof. T. Kokubo and Prof. R. Reis. During her Masters, she worked with Prof. Atsuo Ito, studying the formation of CaP layer on osteoconductive materials by using in situ *atomic force microscopy. Her work has been recognised by the ESB-2003 and by the Bioceramics-2004. Also, her PhD thesis was awarded with the prize of Best PhD Thesis, by the School of Engineering-UM. She is currently working towards a Post-Doc in smart bone tissue engineering scaffolds, enhancing material biofunctionality, at 3B's Research Group-UM.*

mineralization is directed by other charged macromolecules, which may be associated with the self-assembled collagen structures. Although collagen has been considered the most important biopolymer in the regulation of bone structure, it is clearly not the sole source responsible for the regulation of bone mineralization since the majority of the body is composed of collagenous tissues that never mineralize. Thus, the role of the noncollagenous proteins (NCPs) associated with bone is considered to be important in either inhibiting or promoting interactions during crystal nucleation and growth. Some of these proteins are highly acidic, and include proteins that are enriched in aspartic or glutamic acid residues, or phosphorylated serine/threonine.[2]

Because intrafibrillar mineralization does not occur simply by trying to crystallize collagen *in vitro* using supersaturated solutions of hydroxyapatite (crystals only nucleate heterogeneously at the surface of the collagen fibers), it is generally assumed that the collagen substrate does not act alone in directing crystal growth, and that the NCPs found in regions of bone growth play an essential role in calcification due to their ability to bind calcium and their high affinity for collagen. Acidic polypeptide additives used to modify crystal growth of calcium-based minerals have demonstrated a crystallization mechanism that proceeds through a liquid-phase mineral precursor. Various features of the crystals produced *via* this mechanism, such as "extruded" mineral fibers and mineralized collagen composites, have led Olszta and colleagues[3–5] to propose a new and very different view on bone mineralization. They hypothesize that an amorphous, liquid-phase precursor could play a fundamental role in the morphogenesis of calcium-based biominerals. They suggest that the charged polymer acts as a process-directing agent, by which the conventional solution crystallization is converted into a precursor process. This polymer-induced liquid-precursor (PILP) process generates an amorphous liquid-phase mineral precursor to hydroxyapatite which facilitates intrafibrillar mineralization of collagen because the fluidic character of the amorphous precursor phase enables it to be drawn into the nanoscopic gaps and grooves of collagen fibrils by capillary action. Once this highly concentrated phase has infiltrated the fibers, the precursor then solidifies and crystallizes upon loss of hydration waters into the more thermodynamically stable phase, leaving the collagen fibrils embedded with nanoscopic hydroxyapatite crystals.

It is clear, however, that template-driven biomineralization, regulated by a number of extracellular matrix components and the participation of bone cells, plays an important role in the formation of bone. Mineralized tissues, such as bone and shells, can in fact be looked as bioceramic–biopolymer composites made by cell-mediated processes.[6] Their production involves an exquisite level of control both of the spatial regulation of the nucleation and growth of mineral and of the development of micro-architecture during formation of these structures.[6] The key components in such sophisticated mineralized tissues are macromolecules that cells produce and which are subsequently incorporated into the biological material.[7,8] These macromolecules may be involved in a wide variety of functions, such as cell adhesion, ion transport, matrix construction, crystal induction

H. S. Azevedo

Helena S. Azevedo received her B.S. degree in Biological Engineering from University of Minho (Portugal) and her PhD from De Montfort University (UK). Then, she joined the 3B's Research Group at University of Minho as a postdoctoral researcher. In 2006, she was awarded with a Marie Curie fellowship which allowed her to work in the laboratory of Prof. Stupp at the Institute for BioNanotechnology in Medicine (Northwestern University, USA). Currently, she holds a tenure-track faculty position in 3B's Research Group. Her present research is focused on biomimetic matrices, based on polysaccharides and bioactive peptides, formed by self-assembling processes.

R. L. Reis

Rui L. Reis is Director of the 3B's Research Group at U. Minho in Portugal and CEO of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine. He is President and CSO of the company Stemmatters. He is a co-author of 385 ISI cited publications (295 full papers), 120 book chapters, several patents and 6 books. He has an h-factor of 34. He is a member of 12 international societies. He is in the editorial board of most of the journals in his field, being the editor-in-chief of the Journal of Tissue Engineering and Regenerative Medicine (Wiley-Blackwell).

J. F. Mano

João F. Mano received his PhD in Chemistry in 1996 from the Technical University of Lisbon, and currently is teaching at the University of Minho. His current research interests include the development of materials, especially derived from natural-based biodegradable polymers, that can react to external stimuli, or biomimetic and nanotechnology approaches applied to biomaterials to be used in tissue engineering and controlled drug delivery. JFM is author of more than 230 papers in international journals, he has been involved in several national and European research projects and in the organization of scientific events on polymer/materials science and biomaterials/tissue engineering.

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and crystal growth regulation,[7,8] such as the acidic (negatively charged) matrix macromolecules which are intimately involved in biological crystal growth.[7–12] Furthermore, these macromolecules are functionalized with acidic groups such as carboxylic acids, sulfonate and phosphate groups, which allow them to be an effective metal ion chelator to interact with the inorganic matrix.[7]

So, in order to develop biomaterials for replacement and regeneration of bone defects it would be necessary to create an implant with a complex structure, in which features of different length scales can be hierarchically organized, *i.e.*, should mimic the living tissue from mechanical, chemical, biological and functional point of view. In order to achieve such an ambitious goal, it is fundamental to understand the structure and properties of the original hard tissue to be replaced. For instance, it would be desirable to prepare synthetic bone analogues that would match both the chemical and mechanical properties of bone. Such a material could be both load-bearing (with the appropriate modulus, strength, and toughness), yet bioresorbable to allow for the body's own tissue repair processes to regenerate natural bone. Moreover it would be necessarily bioactive. The distinct strategies that have been used to develop bone-like materials, with their achievements and limitations, will be described and discussed in the following sections.

2. Conventional approaches to develop bioactive bone-like materials

The bone-bonding ability of a biomaterial is a very important property for bone tissue regeneration/replacement applications. Hench *et al.*[13,14] have showed for the first time that some glasses, which contain SiO_2, Na_2O, CaO and P_2O_5 in specific proportions, spontaneously bond to living bone. Since then, many bioactive glasses such as Bioglass®,[13,14] bioactive glass ceramics such as Ceravital®,[15] A–W glass-ceramic,[16,17] or dense calcium phosphate ceramics such as synthetic hydroxyapatite (HA)[18,19] have been used clinically with bone-bonding ability. They have been developed in the forms of bulks and particulates with dense and porous structures. For example, Bioglass® in the form of particulates has been extensively used in periodontal bone repair.[20] HA, in bulk and granular forms with dense and porous structures, is currently used as bone spacers and fillers.[21] A–W glass-ceramic has been applied, not only as bone spacer and filler in the bulk and granular forms, but also as artificial vertebrae, intervertebral discs, and iliac crests in dense bulk form.[17] Bioactive glasses have also been found to support enzyme activity,[22,23] vascularization,[24,25] foster osteoblast adhesion, growth, differentiation and induce the differentiation of mesenchymal cells into osteoblasts,[26,27] which are extremely important aspects regarding tissue engineering applications. Particularly relevant for the development of bone tissue engineering was the finding that the dissolution products from bioactive glasses, in particular the 45S5 Bioglass® composition, upregulate the gene expression that control osteogenesis and the production of growth factors.[28] However, even A–W glass-ceramic, which has higher mechanical strength than the other bioactive ceramics and human cortical bone, cannot be used to repair bone defects in high-load bones, such as femoral and tibial bones, as its fracture toughness is lower and its elastic modulus is higher than those of cortical bone.

These bioactive ceramics have the capacity to form a mechanically strong bond with bone when they are implanted through a biologically active hydroxycarbonate apatite (HCA) layer formed on their surface that is chemically and structurally similar to the mineral phase.[13,16,17,29] Such a type of calcium phosphate (Ca–P) layer is not observed around materials that are not bioactive, like metals and polymers when implanted in bone defects, demonstrating that this biologically active bone-like apatite layer is a prerequisite for the bonding between an artificial material and living bone.[15,30]

The analysis of the bioactivity of artificial materials when implanted *in vivo* has been reproduced *in vitro* by immersion experiments using a simulated physiological solution that mimics the typical ion concentrations in body fluids.[31] The human blood is composed of proteins, cells and in terms of inorganic ion species is a highly supersaturated solution with respect to apatite, however it is too complex to reproduce *ex vivo*.[1] Therefore, to understand what is the mechanism of apatite formation in bioactive materials, Kokubo *et al.*[31,32] proposed a protein-free and acellular simulated body fluid (SBF) with pH 7.40 and ionic composition (Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mM) nearly equal to those of the human blood plasma.

It is known that each surface-active ceramic has its own characteristics regarding the formation of the apatite layer. For example, when Bioglass® is soaked in SBF the first reaction of this type of bioactive glass surface is ion exchange, in which Ca^{2+} and Na^+ in the glass exchange with H_3O^+ in the solution, resulting in a pH increase of the solution as well as in the formation of a hydrated silica gel layer.[14,33] The formation of this hydrated silica gel layer at the surface of Bioglass®, which is abundant in silanol (Si–OH) groups, provides favourable sites for the calcium phosphate nucleation.[14,34,35] Furthermore, the water molecules in SBF react with the Si–O–Si bond to form additional Si–OH groups.[36] Then, these functional groups induce apatite nucleation, and the released Ca^{2+} and Na^+ ions accelerate apatite nucleation by increasing the ionic activity product (IAP) of apatite in the fluid. Tanahashi *et al.*[37] have also reported that Si–OH groups were effective in apatite nucleation. Therefore, the mineralization induced by bioactive ceramics is due to the formation of specific surface functional groups such as Si–OH, which serve as effective sites for heterogeneous nucleation of Ca–P.[38] Additionally, an increase of IAP in the surrounding fluid could thereby promote the Ca–P nucleation and growth at the surface of bioactive ceramics.[38]

So, the extensive use of ceramics in the field of bone tissue regeneration and replacement, alone or as a component of a composite, is not only related with the need of developing materials with adequate mechanical strength, but it is undoubtedly due to the bone-bonding ability described in this section, typically presented by this class of materials.

It must be noted that in a real *in vivo* situation achieving an interface that strongly bonds the implant to bone tissue is a great challenge, in particular because we are dealing with two mechanically distinct materials. Until now this challenge has not been fully accomplished, because it is dependent on several complex aspects such as the adhesion strength of the interface,

the resistance to wear or even the biological response at the implant site. Also, *in vivo* musculoskeletal tissues present mechanical gradients at interfaces, which reduce stress concentrations as loads are redistributed. It is known that the most common cause of ligament and tendon grafts is rupture at insertion sites.[39] So, a way to improve their *in vivo* performance could be the insertion of distinct transition zones to improve load transfer between tissues in the future substitutes for orthopaedic applications. Some efforts have been made towards this direction by proposing scaffolds for osteochondral defects with two or more layers with distinct compositions and, hence, with distinct mechanical properties.[40,41] However there is still much to do in order to improve the implant interface *in vivo*.

3. Nanocomposites

The most obvious choice of materials for a synthetic analogue of bone would be a collagen–hydroxyapatite composite. We can say that such a composite would mimic the natural bone matrix that, as described in the introduction, consists primarily of hydroxyapatite nanocrystals deposited in between highly ordered collagen-I fibers. Both components would render the necessary mechanical strength and, in addition, hydroxyapatite would confer the necessary bioactivity to collagen. However, from the research in this area, namely from the attempts to mineralize collagen *in vitro*,[3] it is clear that the collagen–hydroxyapatite composites developed so far, typically with microsized mineral particles, don't reproduce the collagen/mineral structure of bone at the nanoscopic level and don't achieve the high mineral loading that is attained biologically by intrafibrillar mineralization.

More recently, work on nanoglasses/nanoceramics and nanostructured biocomposites have shown that these materials provide alternatives not yet fully explored for orthopaedic applications,[42] presenting improved mechanical and biocompatibility properties and exhibiting, in some extent, a micro- and nanoarchitecture similar to bone.[1,43] When compared with conventional ceramics or glass micro- or macro-particles, the use of nano-sized particles may have advantages in bone repair or regeneration, because it has been shown that the decrease of grain size allows the up-regulation in cellular adhesion, enhances osteoblast proliferation and differentiation and the biomineralization process is also enhanced.[42] Moreover, the use of bioactive nanoparticles may have intrinsic sense in the design of materials for biomedical applications. Fig. 1 shows the possibility of using nanoparticles in the fabrication of materials, organised according to the dimension of the material: fibers (1D); surfaces (2D); and porous and other 3D systems.

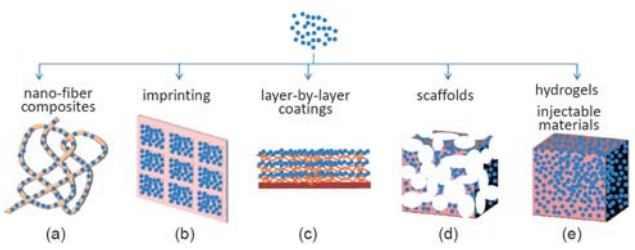

Fig. 1 Strategies related to the use of nanoparticles in the production of bioactive materials: (a) nanocomposites based on the fabrication of nanofibers; (b) spatial control of nanoparticles in the production of patterned bioactive surfaces; (c) bioactive multilayered coatings produced by layer-by-layer; (d) polymer-based scaffolds; (e) hydrogels or solid bioactive biomaterials.

The combination of a polymeric matrix and bioactive nanoparticles may be used to produce nanocomposites composed by nanofibers (Fig. 1a). For example, electrospinning has been used for this purpose, in which hydroxyapatite nanoparticles were utilized.[44–46]

Nanoparticles can also be deposited onto surfaces. The spatial control of the regions where the nanoparticles are dispersed may produce patterned bioactive surfaces (Fig. 1b). Moreover, we can also control the deposition of nanoparticles on surfaces in which the coating thickness may be controlled, using, for example layer-by-layer technology (Fig. 1c). Such methodology has been used to produce multilayered organic–inorganic composite films that included bioactive glass nanoparticles.[47] The obtained bioactive coatings tried to mimic the ordered brick-and-mortar arrangement found in the microstructure of seashell nacre, known for its superior hardness, strength and toughness.[48] Nanoparticles can also be included in 3D composite materials as one may improve the final mechanical properties as compared with the use of larger particles. Bioactive nanoparticles may be included in scaffolds (Fig. 1d); for example, poly(L-lactic acid)-based scaffolds containing bioactive glass nanoparticles, induced the precipitation of apatite onto the surface of the pores upon immersion of SBF.[49] Non-porous materials including nanoparticles in the form of gels or hard devices may also be produced (Fig. 1e). As an example, chitosan–β-glycerophosphate salt formulation with bioactive glass nanoparticles was conceived to prepare novel thermo-responsive hydrogels exhibiting a bioactive character.[50] Such systems are liquid at room temperature and turn to a gel at body temperature, being thus adequate to be used as an injectable system. The use of nanoparticles in this context facilitates the introduction of the liquid *in situ* through a minimally invasive procedure.

It should be noted that until now it was not possible to develop composites that match the complexity of bone tissue. In particular, a critical issue regarding composite implants is the lack of a well-defined interface between their constituents. Due to this feature, these materials exhibit serious mechanical property mismatches with natural bone tissues, which can cause stress shielding and lead to bone resorption when the material has a higher Young's modulus than bone. Very often, revision surgery will be required to follow up the initial implantation. A second major limitation of traditional bone implants is the lack of interaction between these implants and their tissue environment. These materials typically do not bear any functionalities that encourage communication with their cellular environment. These "static" implants are not capable of effectively triggering the healing cascade upon surgical implantation, therefore limiting the potential for tissue attachment and in-growth.

Nevertheless, many bone tissue substitutes have been developed and some are already used in clinical trials or as already approved therapies. Besides the examples already given we can also mention some already approved bone substitutes, such as the Vitoss scaffold FOAM from Orthovita, composed of bovine type I collagen and β-TCP available since 2004 and the FortrOss from Pioner Surgical, available since 2008, composed of

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nanocrystalline hydroxyapatite and a copolymer of porcine collagen and dextran, both used to treat bone injuries.

4. Designing functionalized surfaces to render biomaterials self-mineralisable

The knowledge about the biomineralization process in natural mineralized tissues and the fundamental findings on bone-like apatite formation on bioactive ceramics, previously described, have provided a platform for developing a new class of bioactive materials as bone substitutes. Some of the innovative strategies to render biomaterials self-mineralisable will be discussed in the following sections.

Research in the area of biomimetic synthesis has been mainly based upon the premise of surface functionalization. The functionalised surfaces are believed to be analogous to nucleation proteins in biological systems in what concerns to provide energetically favourable interfaces for heterogeneous nucleation and growth of inorganic films from supersaturated solutions.[2,51,52]

In the last decade several strategies have been employed for the development and investigation of new functional groups for apatite nucleation. For the readers, it is important to be always in mind that the ideal implant should present a surface conductive to or that will induce osseointegration, regardless of the implantation site, bone quantity, bone quality, *etc.*[53] Besides the Si–OH groups referred in section 2, other functional groups have been shown to induce bone-like apatite formation, namely Ti–OH, Zr–OH, Nb–OH, Ta–OH, –COOH, and PO_4H_2.[36] All these functional groups have isoelectric zero points at pH values much lower than 7 and, thus, are negatively charged in the living body,[54] inducing apatite formation through formations of an amorphous calcium compound, *e.g.*, calcium silicate, calcium titanate, and the subsequent formation of an amorphous calcium phosphate.[36] This calcium phosphate spontaneously transforms into apatite, the stable phase in body environment.[55]

Understanding the surface chemistry and knowing the main mechanisms responsible for induction of apatite formation provided very important tools to design new bioactive materials. Furthermore, bioactivity can be induced on surfaces that are not bioactive by themselves, either by the incorporation of functional groups or by forming thin ceramic phases that have the potential to form functional groups upon exposure to a body environment.[32,36,56] The key point lies in the design of an organized functionalized surface to control the mechanisms of heterogeneous nucleation. Several examples of these promising bioactive materials can be found in literature such as tough bioactive metals, soft bioactive inorganic–organic hybrids and bioactive inorganic–organic three-dimensional composites with a bone-like structure.[36,57,58] For example, Kim *et al.*[59] demonstrated that heterogeneous nucleation and growth of a bone-like apatite layer can be induced by hydroxylation of metal oxide surfaces placed in SBF for different periods of time: the formed Ti–OH groups induced the apatite formation on it, through formation of an amorphous calcium titanate and amorphous Ca–P.

In the case of organic surfaces there is an advantage that is the capacity to tailor their surface to achieve different properties such as making their surfaces more hydrophilic and capable of carrying functional groups. In addition, these materials have much higher degree of structural flexibility and may have strong surface-specific binding forces, such as the ability of the functional groups to chelate metal ions.[60] Therefore, the new strategies aim to tailor material's surface not only to render the materials biologically active, but also to preserve the bulk properties of the underlying substrate. One true analogue of biomineralization would be a polymer matrix which can be placed into a metastable solution and induce precipitation to occur within the polymer but not in the solution.[60]

Tanahashi and Matsuda[61] have shown that the incorporation of bihydrogenophosphate ($–PO_4H_2$) and carboxyl (–COOH) groups on self-assembled monolayers (SAMs) are effective for apatite nucleation but not the amide ($–CONH_2$), hydroxyl (–OH), amine ($–NH_2$) and methyl ($–CH_3$) groups. Similar work was developed by Leonor and co-workers[62,63] where the incorporation of acid groups onto the polymer surfaces, namely sulfonic ($–SO_3H$) groups, could also serve as effective functional groups for apatite nucleation (Fig. 2).

Murphy and Mooney[64] reported that the process of mineral growth on biodegradable polymers can be augmented and controlled by variation in the functional groups present at the mineral nucleation site or the ionic characteristics of mineral growth environment. Polymer surface functionalization was achieved through hydrolysis of poly(lactide-co-glycolide) (PLGA), which results in an increase in the amount of surface carboxyl and hydroxyl groups due to scission of polyester chains. The presence of these groups regulates the calcium binding to the polymer surface and the heterogeneous mineral growth. Similar results were obtained by Oyane *et al.*,[65] where bone-like apatite was formed at the surface of poly(ε-caprolactone) (PCL) porous scaffolds in SBF, previously treated with aqueous NaOH, which introduced carboxyl groups, and then dipped alternately in calcium and phosphate ion solutions to induce apatite nucleation. However, this treatment required a long time period to induce apatite nucleation in SBF and the need to be combined with calcium ions. Therefore, the same authors demonstrated[66] that when PCL is previously treated with O_2 plasma, and then dipped alternately in alcoholic solutions containing calcium ions and phosphate ions, a bone-like apatite layer was formed at the surfaces of PCL plates and PCL 3D meshes in SBF within 24 h.

An apatite–polymer fiber composite would be a good candidate for a bioactive material with analogous mechanical properties to those of living bone.[67] So, it was proposed that such a type of composite could be synthesized, if the organic fibers would be arranged in a 3D structure similar to that of collagen fibers in living bone, and if they would be modified to contain effective functional groups for apatite nucleation onto their surface.[67] Oyane *et al.*[68,69] successfully produced bioactive films textured on the 3D-templates of polymers by functionalization, coupling and hydrolysis of iso-cyanatopropyltriethoxysilane or sol–gel coupling of calcium silicate on ethylene-vinyl alcohol (EVOH) polymer. Balas *et al.*[70,71] demonstrated that by treating organic polymers, namely polyethylene terephthalate (PET), EVOH and Nylon 6, with a silane-coupling agent and a titania solution, they were able to induce the formation of bone-like apatite in SBF.

In the case of polysaccharides, such as carboxymethylated chitin[67] and gellan gum gels,[67] it is possible to induce apatite formation by subjecting them to a very simple alkaline treatment.

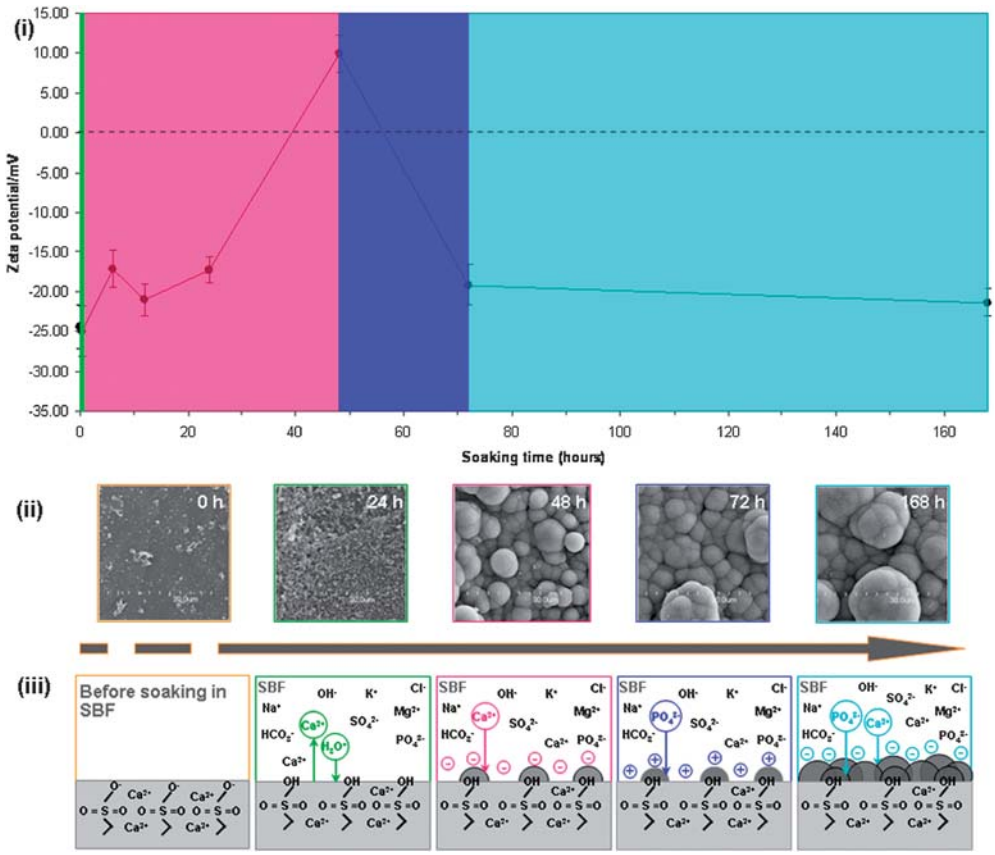

Fig. 2 The relationship between the changes in the surface structure and the potential of the incorporation of $–SO_3H$ groups into HMWPE in the apatite formation process on its surface in SBF: zeta potential (i) and SEM photographs (ii) of the sulfonation and $Ca(OH)_2$ treated HMWPE as a function of soaking time in SBF. The mechanism of apatite formation on bioactive polyethylene in SBF is due to electrostatic interaction of the polymer surface and ions in the fluid, which progresses in the following way (iii): formation of $–SO_3H$ groups with a negative charge by the Ca^{2+} ions release from the HMWPE sample; formation of an amorphous calcium sulfate with a positive charge by combination of negatively charged $–SO_3H$ with the positively charged Ca^{2+} ions in the SBF; formation of an amorphous calcium phosphate by combination of the positively charged calcium sulfate with the negatively charged phosphate ions in the SBF; and formation of the apatite with a negative charge by transformation of the calcium phosphate into crystalline apatite. Data adapted from the results of ref. 63.

Kawashita *et al.* found that by soaking these gels first in saturated calcium hydroxide ($Ca(OH)_2$) solution and then in SBF, they become bioactive. This was attributed to the catalytic effect of the carboxyl groups present on both materials on apatite nucleation and the acceleration of this process due to the release of Ca^{2+} ions. However, in the case of curdlan gels,[67] which present hydroxyl groups, an apatite deposit was not formed even after the $Ca(OH)_2$ treatment. Such results provide a fundamental condition for obtaining an apatite–polymer fiber composite with analogous structure to living bone by using a biomimetic method.[67] Similar research works have been reported by other authors,[72,73] where carboxymethylated chitin and chitosan were able to induce apatite formation.

Also, Kokubo *et al.*[74] showed that carboxymethylation of chitin non-woven fabric treated with a saturated $Ca(OH)_2$ aqueous solution induced the formation of an apatite layer within 3 days in SBF. This kind of composite can be useful as a flexible bioactive bone-repairing material.

Starch-based polymers such as corn starch with ethylene-vinyl alcohol (SEVA-C), an organic and quite hydrophilic material, may be a suitable material for inducing apatite nucleation, as in fact biological mineralization is thought to be induced by anionic functional groups. SEVA-C, can associate a degradable behaviour with an interesting mechanical performance.[75,76] However, in terms of bone bonding, this polymer cannot induce by itself the formation of an apatite layer without a previous bioactive coating or the use of bioactive fillers as it has been reported previously.[77,78] The presence of reactive –OH groups on starch and vinyl alcohol justifies the efforts in trying to incorporate other polar groups such as –COOH groups in order to obtain bioactive polymers. For that purpose, a new route was developed for the surface functionalization of biodegradable polymers,[79] in which two different types of alkaline solutions, calcium hydroxide solution ($Ca(OH)_2$) and sodium hydroxide solution (NaOH), were used. This method is based on a wet chemistry modification, resulting in etching and/or hydrolysis in order to increase the amount of polar groups such as hydroxyl (–OH) and carboxylic (–COOH) groups on the surface of the polymer.

Very similar results were also obtained in our research group, with starch based blends after surface oxidation.[80] As mentioned above, starch itself contains many –OH groups (non-ionic). In order to alternate non-ionic starch hydroxyl groups with

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negatively charged carboxyl groups, a surface oxidation by potassium permanganate ($KMnO_4$)/(NHO_3) nitric acid system was performed.[80] The formation of an apatite layer was observed in different blends of starch and synthetic polymers. The $KMnO_4/NHO_3$ oxidizing system has been shown to be a very straight-forward applicable method for introducing polar groups on starch based biomaterials.

These results suggest that these rather simple treatments are efficient methods for surface functionalization and subsequent mineral nucleation and growth on biodegradable polymers to be used for bone related applications. On the other hand, it is important to comment that even some materials that contain carboxyl groups on their structure before any treatment do not form apatite on their surfaces after immersion in SBF. This indicates that the catalytic effect of these functional groups is not strong enough to induce apatite nucleation by itself.[67]

All the studies shown here share a common finding in which a surface with an organized arrangement of functional groups can act as a template for the biomimetic growth of apatite. On the basis of this research several kinds of bone-bonding material with different mechanical properties can be developed in the future.

5. Smart mineralizing surfaces

Many examples exist in the area of biology/materials science interface where polymers that react reversibly to external stimuli are used in systems designed to respond to specific environmental changes, with biological applications that include drug delivery, cell culturing or tissue engineering/regenerative medicine.[81,82] The use of these so-called stimuli-responsive polymers has also been proposed to introduce a smart character in the control of biomineralization.[83,84] In these works the surface of bioactive substrates, composed by poly(L-lactic acid) reinforced with Bioglass®, was modified by coupling either poly(*N*-isopropylacrylamide) (PNIPAAm),[83] a thermo-responsive polymer, and chitosan, a pH-responsive polymer,[84] using plasma activation. It was shown that surface biomimetic mineralization may be triggered by these two types of stimuli: a temperature change[83] or a pH change.[84]

PNIPAAm is the most studied synthetic thermo-responsive polymer and exhibits a lower critical solution temperature (LCST) at about 32 °C in aqueous solution, changing sharply from a hydrophilic to a hydrophobic state upon heating.[85] It is believed that this transition involves the breakage of intermolecular hydrogen bonds between the water molecules and the amide groups in the polymeric chains, which are replaced, above the LCST, by intramolecular hydrogen bonds amongst the dehydrated amine groups. The thermo-responsive nature of the modified composites was easily confirmed by contact angle measurements. The water contact angle for the PNIPAAm modified PLLA + 10% BG film was 51.9 ± 2.4°, at 25 °C whereas at 37 °C it changed to 58.8 ± 2.4°, being consistent with the increase in the hydrophobicity of the surface above the LCST.[83] It was found that these conformational changes occurring at the surface influence the apatite formation of the bioactive composites below and above the LCST after being immersed during 2 weeks in SBF. In fact, for the PNIPAAm modified composite film with 10% BG no apatite formation could be observed at 25 °C (Fig. 3). However, at 37 °C the treated film could form dense precipitates with the typical cauliflower morphology, containing needle-like nanometric structures, characteristic of biomimetic-formed apatite (Fig. 3).

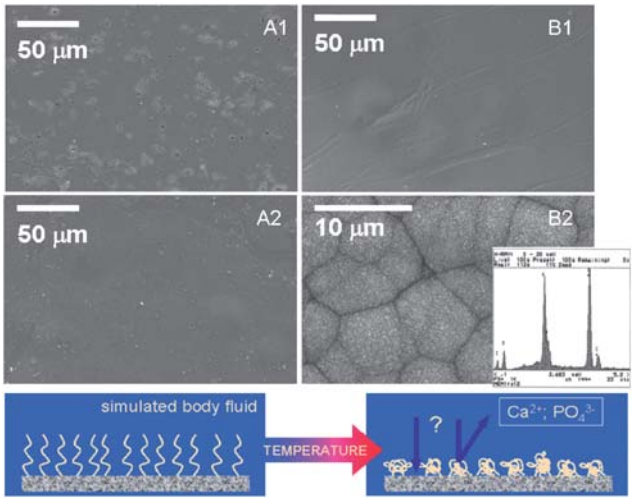

Fig. 3 SEM images of unmodified (1) and PNIPAAM grafted (2) films with 10% of BG after immersing in SBF during 2 weeks at 25 °C (A) and 37 °C (B). The inset picture corresponds to the EDS spectrum of the CaP coating formed at 37 °C. A scheme is also shown representing the different conformational states of the PNIPAAm chains at both temperatures. Data adapted from the results of ref. 83.

Chitosan is a pH responsive polymer that contains both hydrophobic ($-CH_3$) and hydrogen bonding favouring groups (–OH, $-NH_2$ and –C=O). In an acidic medium this polymer becomes positively charged due to the protonation of the free amine groups (the pK_a is ~6)[86] and polymer–polymer interactions *via* hydrophobic effect and/or hydrogen bonding junctions can be hindered due to electrostatic repulsion.[87] The pH-responsive behaviour of the composites modified with this smart polymer was analysed by contact angle measurements.[84] The unmodified PLLA/BG films revealed a quite hydrophobic character, presenting a contact angle of 82°, independently of the pH.

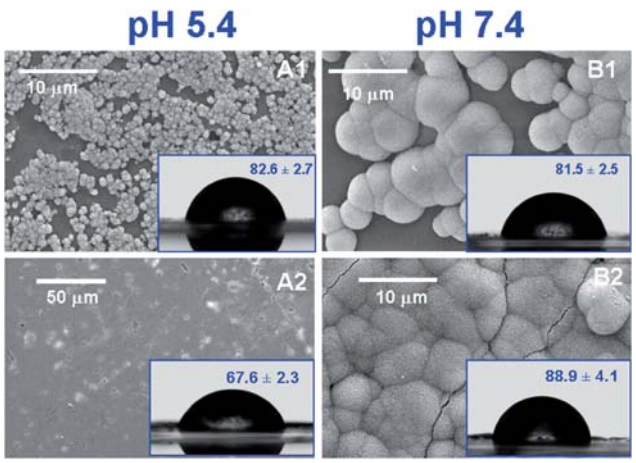

Fig. 4 SEM images of unmodified (1) and chitosan grafted (2) films with 30% of BG after immersing in SBF during 3 weeks at pH 5.4 (A) and pH 7.4 (B). The inset images show the water contact angle measurements for the referred materials. Data adapted from the results of ref. 84.

After modification, the contact angle changed from 88.9° ± 4.05° at pH 7.4 to 67.6° ± 2.3° at pH 5.4 (Fig. 4). The apatite formation on the surface upon immersion of the modified films in SBF was investigated at pH 5.4 and pH 7.4 by SEM.[84] It was found that such modification, together with the effect of pH, could block the formation of apatite onto the biodegradable substrate when the pH changed to 5.4 (Fig. 4). On the other hand, a dense apatite layer was formed at pH 7.4 (Fig. 4). For the unmodified substrates an apatite layer was formed at both pHs (Fig. 4).

Energy dispersive spectroscopy (EDS), thin-film X-ray diffraction (TF-XRD) and Fourier transform infrared spectroscopy (FTIR) analysis of the coatings formed at 37 °C confirmed the formation of a carbonated apatite mineral similar to the major mineral component of vertebrate bone tissue.[83,84] Although in the above mentioned works temperature and pH were chosen, this concept of smart apatite formation can obviously be extended for other source of responsiveness and for other kind of mineral deposition.

Moreover, by patterning the modification of the surface, it was possible to combine stimuli (temperature in this case) and spatial control of biomimetic apatite formation.[83] This was achieved by just exposing some regions of the substrate surface to the plasma treatment, allowing the insertion of PNIPAAm in specifically desired areas. Again, no apatite formation was observed for these modified films at 25 °C after 2 weeks immersion in SBF.[83] However, apatite aggregates were formed at 37 °C, with a circular shape and randomly distributed over the composite surface, being consistent with the PNIPAAm patterning generated during the plasma activation step.[83] Other apatite patterns could be produced (*e.g.* rows, squares, grids) just by changing the mask model or using other lithographic methodologies. It is known that apatite-coated surfaces enable the attachment, growth and expression of osteogenic genes in osteoblasts-like cells.[88,89] Thus, these apatite patterned surfaces could be used in fundamental studies on differentiation, adhesion, proliferation and cell–cell signaling of bone-related cells. These surfaces could also be used in fundamental co-culture studies involving bone cells and other kind of cells such as endothelial cells, which could be useful in bone tissue engineering applications.

6. Template-driven mineralization

Crystal growth habit can be modified when the relative order of surface energies can be changed or when crystal growth along certain crystallographic directions is selectively hindered by a crystal growth modifier. In the presence of crystal growth modifiers, the preferential/selective adsorption of crystal modifiers to a specific crystallographic face becomes stronger than that of others due to the anisotropy in adsorption stability decreasing the surface energy of the adsorbed face and inhibiting the crystal growth perpendicular to this face, thus altering the final shape of the crystal. In addition, the crystal shape can be altered if the growth process occurs in a confined environment.[90] The topography of the substrate onto which the crystals are growing may also influence its morphology; this was suggested, for example, in an apatite precipitation study onto flat or textured poly(L-lactic acid) surfaces, in a 3D environment.[91]

The morphological control exerted in biomineralization may be separated into a three-component system:[5] (1) an insoluble organic matrix, that can play a role in the compartmentalization of the growing mineral and/or templating the nucleation for controlled crystallographic orientation and/or phase; (2) soluble acidic proteins that are frequently occluded within the crystal and are thought to play a role in the control of crystal shape; (3) vesicular compartments that provide spatial and temporal control of ion and additive transport to the mineralization front. Although strategies mimicking nature have partially succeeded in synthesizing bio-inorganic composite materials, our limited understanding of fundamental mechanisms has so far kept the level of hierarchical complexity found in biological systems out of materials engineer's capabilities. Different approaches have been used to control the morphology, microstructure and complexity of inorganic materials with two and three dimensionalities, including mineralization on artificial interfaces (self-assembled monolayers), natural and synthetic matrices/templates for controlled crystal growth, and emerging crystallization on patterned surfaces for the creation of patterned crystals. These approaches have been, however, extensively studied for calcium carbonate systems.[90,92]

Inorganic materials can be artificially structured at different length-scales using biomineralization and templating approaches. Crystal growth mimicking biomineralization has been studied using various kinds of organic molecules and molecular assembly.

As a first step towards the design and fabrication of biomimetic bonelike composite materials, Song and co-workers[93,94] have developed a template-driven nucleation and mineral growth process for the high-affinity integration of hydroxyapatite with a poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogel. The described mineralization method takes advantage of the different solubilities of hydroxyapatite in acidic and basic media and the chemically labile nature of the ester groups of pHEMA in basic media. There are several notable features of this procedure. First, increasing pH and temperature during the process promotes the hydrolysis of the ethyl ester side chains of pHEMA and leads to the *in situ* generation of an acidic surface and a partially acidic interior that has high affinity for calcium ions, promoting the nucleation and growth of calcium phosphate on the surface, along with extensive calcification of the hydrogel interior.

Molecular self-assembly exhibits a number of useful properties, including the possibility of creating synthetic systems with high order parameters and can offer the potential for epitaxial events in synthetic systems that emulate protein-mediated mineralization.

A designed hierarchical structure was made by the self-assembly of nanofibrils of mineralized collagen resembling an extracellular matrix.[95] The collagen fibrils were formed by the self-assembly of collagen triple helices. Hydroxyapatite crystals grew on the surface of these fibrils in such a way that their c axes were oriented along the longitudinal axes of the fibrils. The mineralized collagen fibrils aligned parallel to each other to form mineralized collagen fibers.

Antonietti *et al.*[96] described a biomimetic approach for the precipitation of unusual morphological forms of calcium phosphate minerals. Double-hydrophilic block copolymers consisting of a long poly(ethylene oxide) block and a short poly(methacrylic acid) block, modified by partial alkylation with dodecylamine (PEO-b-PMAA-C12) were employed as templates for the

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controlled precipitation of calcium phosphate from aqueous solution at different pH values. They showed that supramolecular preorganization of these water-soluble double hydrophilic block copolymer can be achieved by hydrophobic modification within the poly(methacrylic acid) domain. This strategy increases the density of functional groups within the aggregate and hence the localized level of supersaturation attainable by metal-ion sequestration. The polymer micelles act as interactive templates where the organic/inorganic superstructure can range between nested clusters of fine nanofibers to compact mesostructures in which nanoscaled calcium phosphate entities are interspersed with ordered polymer domains.

Hartgerink *et al*. have designed a peptide molecule, designated a peptide amphiphile, that self-assembles into cylindrical nanofibers (~7 nm in diameter) upon screening of charged groups due to changes in pH, or addition of multivalent ions. This peptide amphiphile consists of a long alkyl tail, which conveys hydrophobic character to the molecule, and a peptide segment, which is its hydrophilic block and includes a phosphorylated serine residue. Once self-assembled, these negatively charged phosphorylated serine residues are displayed near the fiber exterior, which are able to interact strongly with calcium ions and help direct mineralization of hydroxyapatite. They observed that the growth of hydroxyapatite crystals was crystallographically aligned with the fibers' long axes. This alignment is the same as that observed between collagen fibrils and hydroxyapatite crystals in bone.

However, mimicking the natural "templating" of bone mineralization may require more than just providing a physical template for calcium phosphate nucleation. It is expected that both spatial and temporal elements are necessary to achieve biomimetic mineralization in synthetic materials.

In a recently published paper, Spoerke *et al.*[98] describe an artificial, *in vitro* biomineralization process that utilizes a nanofiber gel as a 3D substrate for biomimetic hydroxyapatite mineralization. The system employs the natural enzyme alkaline phosphatase (ALP) and a phosphorylated, anionic nanofiber gel matrix to template, in 3D, hydroxyapatite nanocrystals. The nanofiber surfaces are strongly enriched with negatively charged and phosphorylated aminoacid residues, which are expected to be densely decorated with calcium ions bound during gelation (Fig. 5a). These "premineralized" surfaces can act as extremely favorable sites for heterogeneous nucleation of apatite crystals. The calcium aggregates and early calcium phosphate seed complexes being exposed to other reactive ions in the surrounding aqueous environment continue to grow and crystallize, eventually forming relatively isolated aggregates of crystals.

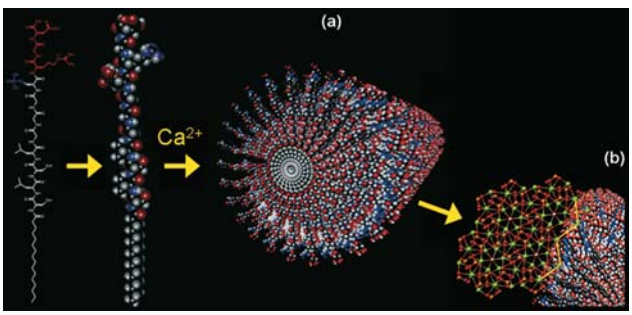

Fig. 5 Schematic illustration of peptide amphiphile (PA) self-assembly into a cylindrical nanofiber upon addition of calcium ions (a). A visualization of a HA crystal nucleating off calcium ions spaced 5.46 Å apart on the PA nanofiber. The HA crystal is shown with the c-axis parallel to the long axis of the PA nanofiber (b). Erik D. Spoerke, Shawn G. Anthony and Samuel I. Stupp, Enzyme Directed Templating of Artificial Bone Mineral, *Adv. Mater.*, 2009, **21**, 425–430. Copyright Wily-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Secreted by osteoblasts, ALP liberates phosphates necessary for hydroxyapatite mineralization from organic phosphates such as β-glycerolphosphate. Enzymatic release of phosphate ions by ALP regulates the availability of the mineral precursor and thus the rate of nanocrystal nucleation. This regulation prevents uncontrolled mineral precipitation, biasing the system toward selective, heterogeneous nucleation on the phosphorylated peptide nanofiber templates. The gradual nature of this enzymatic process provides critical regulation of free phosphate concentration, preventing rapid, uncontrolled nonspecific mineralization in the incubating medium. Simple introduction of free phosphates to the mineralizing environment produced relatively uncontrolled calcium phosphate formation. In contrast, when β-glycerophosphate was used instead of free phosphates, the phosphate needed for reaction had to be harvested by the ALP in solution. The enzyme-mediated release of these phosphates was sufficiently slow and the solution never became sufficiently supersaturated with respect to phosphates to allow spontaneous nucleation of calcium phosphates in solution. Rather, as phosphates were cleaved from their organic counterparts, they were consumed in the mineralization processes localized on the calcium-laden nanofibers.

The chemistry of these self-assembled synthetic nanofibers simultaneously provides strong hydroxyapatite nucleation sites, enriched with local calcium concentrations. What makes this system distinct from other templating designs was the use of ALP to moderate phosphate introduction to the system. Utilizing ALP to regulate the slow introduction of enzymatically-liberated phosphates to the system was fundamental to provide sufficient phosphates for mineralization in a time frame that was appropriate for specific, nucleation and templated growth of hydroxyapatite on the nanofibers (Fig. 5b).

Inspired by nature, researchers have made enormous progress over the last few decades in mimicking some of the key structural and biochemical functions of bone. However, because the natural environment of bone tissue is extremely complex to recreate, none of the current materials imitate the highly organized structure of mineralized tissues. So far, efforts to produce biomimetic minerals on organic matrices have focused mostly on design of the structural template, using collagen, peptides, and polymers. Understanding the biological processes involved in bone mineralization is and will be of great importance to design materials for bone regeneration. Specifically, a quantitative understanding of the local concentration, distribution, and interaction of key molecules within normal, diseased, and regenerating bone, as they change with time, is important to be able to adequately create bone-like materials. For example, the calcium and phosphate concentrations to promote apatite mineralization in bone are controlled by cellular activities, which are difficult to recreate. In the authors' opinion, the most obvious

materials to mimic bone mineralization will be biomaterials that can instruct cells to produce the complex integration of mineral and organic phases that is achieved in human bone and be able to trigger its regeneration *in vivo*.

7. Conclusions

Biomineralization processes result in organic/inorganic hybrid materials with complex shape, hierarchical organization and superior materials properties. Chemistry, which is inspired by these processes, aims to mimic biomineralization principles and to transfer them to the general control of crystallization processes. However, the principal limitation of the current bio-inspired bottom-up mineralization approaches is that they can only yield self-assembled structures up to the micrometre level, unless an external template is provided. Higher structural levels that can be found in biominerals are controlled by cell action, which so far does not have a close synthetic mimic. Therefore, only structured templates like patterned monolayers, biomineral replicas, or the original structural biomineral matrix can be applied to achieve a structuration up to the macroscopic scale. Nevertheless, despite these limitations, the study of bio-inspired mineralization and self-assembly processes can help to understand parts of the bone mineralization and explore ways in which biomineralization principles can be used for the synthesis of advanced biomaterials. Mimicking the structure of natural bone, even with very simplified synthetic systems, has already led to remarkable results concerning the generation of complex mineral morphologies and control over crystallization events. However, most of the crystallization mechanisms are still unknown due to the complexity of the system, which involves time-dependent structures with sizes spanning the entire colloidal level. In this way it is certain that there is still much to be learned from the way nature assembles its many biologically important structures.

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