ADVANCED NANOBIOMED RESEARCH
www.advnanobiomedres.com

# Engineering of Extracellular Matrix-Like Biomaterials at Nano- and Macroscale toward Fabrication of Hierarchical Scaffolds for Bone Tissue Engineering

*Rafael Lemos, F. Raquel Maia, Rui L. Reis, and Joaquim M. Oliveira**

**The increasing rate of musculoskeletal pathologies has compelled the development of improved and novel treatment strategies in order to address unmet clinical needs. Tissue engineering approaches comprising the use of scaffolds for bone regeneration have been showing to be a promising alternative to conventional bone repair/substitution approaches. In particular, hierarchical scaffolds as methods of structural support and osteogenic differentiation promoters are among the most used tools in bone tissue engineering (BTE). In this reasoning, hierarchical scaffolds have sparked the field, striving toward mimicking the natural bone tissue in both, its complex 3D structure and composition. A recent and promising trend has been the merging of nanotechnology and tissue engineering concepts. As such the incorporation of nanoparticles and nanocomposites into micro- or macroscaffold systems can result in an improvement of scaffolds' biofunctionality at different levels. These tools are versatile in nature and can be used for multiple purposes such as drug delivery, thermal conductors, and mechanical reinforcement. Taking into consideration multidisciplinary approaches, several strategies have been pursued. The recent reports dealing with the approaches pursued in the hierarchical scaffolds production and enhancement, ranging from the nanoscale to the macroscale, are overviewed herein.**

## 1. Introduction

With the increasing rate of musculoskeletal pathologies affecting the world population due to ever-higher life expectancy and the aging population, the development of better treatment strategies for tackling bone-related disorders is of great importance. Bone tissue engineering (BTE) presents an important and promising alternative to conventional approaches such as the use of autografts, considered the current gold standard treatment.[1] The use of top-down techniques is the most typical and allows for the use of a wide range of materials and good control over the scaffolds' physical properties.[2] Furthermore, to be an efficient tool, a scaffold should be biocompatible, nontoxic, provide structural support for cell attachment, and support in vivo development of extracellular matrix (ECM) in damaged tissue. The bone's ECM possesses hierarchical organization and plays an important role in fracture repair.[3] It is mainly composed of collagen type I and II, elastin, fibronectin, laminins, proteoglycans, glycosaminoglycans, and growth factors bond to the ECM proteins to be released according to the physiological needs of the cell.[4] Among the scaffolds developed, biomimetic hierarchical scaffolds have sparked the development of improved BTE approaches.[5,6] Mimicking the native tissue in its hierarchical structure, chemical composition, and properties is the ultimate goal of regenerative scaffold designing. In BTE, the strife for ever more biomimetic scaffold systems has led to use biomotifs in scaffold design, attempting to recreate bone matrix structure and composition.[7] There are several different approaches and targets for biomimicry. Striving toward mimicking the natural bone tissue in respect to both complex 3D structure and composition is a step forward in biomaterial research but also toward developing patient-specific therapeutics.[8,9]

In recent years, several BTE approaches have been developed that resulted in scaffold systems that more closely mimic the natural tissue.[10] Several different strategies can be used to mimic specific aspects and properties of bone and its ECM. The mimicking of specific structural, mechanical, and biological cues of the ECM that can facilitate host cell differentiation and proliferation is achievable using BTE approaches.[11]

R. Lemos, F. R. Maia, R. L. Reis, J. M. Oliveira
3B's Research Group
I3Bs – Research Institute on Biomaterials, Biodegradables and Biomimetics of University of Minho
Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine
AvePark, Parque de Ciência e Tecnologia, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal
E-mail: miguel.oliveira@i3bs.uminho.pt

R. Lemos, F. R. Maia, R. L. Reis, J. M. Oliveira
ICVS/3B's – PT Government Associate Laboratory
Braga/Guimarães, Portugal

R. Lemos
Centre of Physics (CFUM)
University of Minho
Campus de Gualtar, 4710-057 Braga, Portugal

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/anbr.202100116.



**DOI: 10.1002/anbr.202100116**

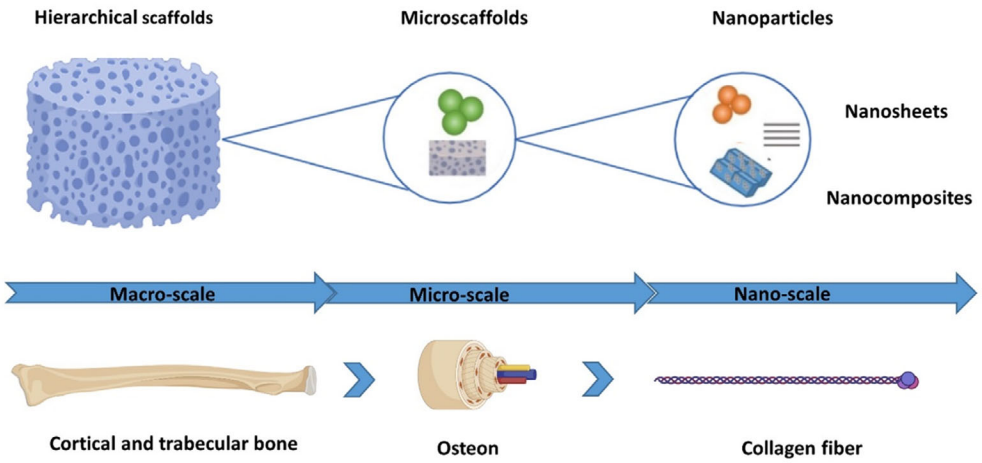


**Figure 1.** Hierarchical bone organization and schematic representation of multiscale approach toward production of hierarchical scaffolds. From nanometric approaches to micrometric approaches to produce a hierarchical scaffold.

The design of hierarchical scaffolds heavily relies on the specific properties of the featured biomaterial. The recent materials of interest and top-down approaches pursued by hierarchical scaffolds' production and enhancement, ranging from the nanoscale to the macroscale (**Figure 1**), are overviewed herein.

## 2. Macrometric Approaches

BTE heavily relies on the development of scaffolds for effective bone defect reparation. The vast majority of the submacrometric approaches complement or enhance developed scaffolds. Scaffolds play a crucial role in BTE by providing a 3D environment for cell adhesion and proliferation.[12] Several biomaterials have proven to be excellent base materials for hierarchical scaffold production as depicted in **Table 1**.

Among these, natural and synthetic polymers, bioceramic scaffolds, and decellularized ECM (dECM) approaches have gained increasing interest in recent years (**Figure 2**).

### 2.1. Natural and Synthetic Polymers

Natural polymers as base materials for bone scaffolds possess the advantage of ensuring bioactivity, a biomimetic surface, and providing optimal cell attachment and growth. Yet, immunogenic response, poor mechanical properties, and low tunability represent challenges for their application.[13,14] Among the most studied natural polymers for scaffolds' production are collagen, silk fibroin (SF), alginate, chitosan, and hyaluronic acid.

Collagen is the main component of the ECM of bone tissue and a natural choice for BTE scaffolds. Collagen is one of the most used biomaterials for scaffold production because it serves as a template for mineralization and it is enzymatically biodegradable. Collagen type I is the most used for engineering applications because it is part of the natural ECM and thus eliminating immunological reactivity.[15,16] For example, Yu et al.[17] used intrafibrillar mineralized collagen hydroxyapatite (HAp) as its building blocks to obtain biomimetic scaffolds produced by a one-step biomineralization process with the help of synthetic analogues of noncollagenous proteins. These scaffolds closely resemble bone in both structure and composition. In this study, Fe and Mn were incorporated in the lamellar scaffold which led to a further improvement of the osteogenic potential of the system. In vitro studies performed with bone marrow-derived mesenchymal stem cells (BMSCs) showed increased osteogenic-specific gene expression and alkaline phosphatase (ALP) activity as well as enhanced cell adhesion and osteoblast proliferation. Additionally, an in vivo assay performed on fresh bone marrow cells showed superior bone regeneration capability of the system.[17]

In the case of SF, its unique mechanical properties make it a versatile tool for multiple applications. In the case of scaffolds' production, this biopolymer presents specific advantages in both versatility and mechanical strength. SF porosity can be controlled through ice-templating allowing the production of anisotropic or hierarchical structures.[18] Moreover, it can be used in a vast array of applications, such as load-bearing scaffolds or sutures, taking advantage of the SF flexibility and high tensile strength.[19] Also, its mechanical strength is superior when compared to other biodegradable polymers, like collagen or chitosan, and its proteolytic degradation presents an advantage when compared to synthetic polymers.[20] The usefulness of these characteristics can be seen in the application of Liu et al.[21] who developed a biomimetic porous SF/biphasic calcium phosphate (BCP) scaffold, using phase separation method and freeze-drying, with improved mechanical properties. The addition of SF improved scaffolds' mechanical properties when compared to pure BCP scaffolds and the incorporation of SF showed to be better for cell growth of preosteoblasts cells. This system that can effectively mimic the natural bone matrix displayed excellent osteogenic capacity in a rat model, as evidenced by the rapid proliferation of seeded osteoblasts and ALP activity. Furthermore, the formation of an apatite layer can occur through the exchange of calcium and phosphorous between BCP and body fluids providing osteoblast with the raw materials to secrete bone matrix.[21]

Alginate is a cytocompatible, negatively charged polysaccharide that has highly tunable mechanical and biological properties.

**Table 1.** Macrometric approaches used in BTE. Hap, hydroxyapatite; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; PLGA, poly(lactic-*co*-glycolic acid); ECM, extracellular matrix; TCP, tricalcium phosphate; 3D, three-dimensional; dECM, decellularized extracellular matrix.

| Category | Type of material | Fabrication methods | Scaffold strategies | Advantages of material | Disadvantages of material | References |
|---|---|---|---|---|---|---|
| Natural and synthetic Scaffolds | Collagen | One-step biomineralization process with help of noncollagenous proteins | Cellular or lamellar structured scaffolds | Incorporates natural bone elements<br>Structure that enhances cell adhesion and proliferation. | Low mechanical strength and osteoinductivity | [17] |
| | Silk fibroin | Phase separation method Freeze drying | Composite-based scaffolds | Optimal mechanical properties<br>Mimicking of natural bone and/or ECM structure | Weak osteogenic capacity<br>Degradation products impact cell activity | [21,52,77] |
| | Alginate | Partial cross-linking with TEMPO-oxidized cellulose nanofibrils forming hydrogel | Bioprinted and biomineralized scaffolds | Optimal mechanical properties<br>Incorporates natural bone elements | Possesses weak natural viscoelastic properties | [23] |
| | PLGA | Layer-by-layer deposition | Layer-by-layer surface coated scaffolds | Optimal mechanical properties<br>Promote mineralization | Hydrophobic nature affects the adsorption of proteins.<br>Deposition of apatite on polymers at nano- and microscale remains a challenge | [27] |
| | Chitosan | In situ precipitation technique | Composite-based scaffolds | Mimicking of natural bone and/or ECM structure | Possesses high flexibility and therefore is not ideal for BTE applications | [25] |
| Bioceramic scaffolds | TCP | 3D plotting technique | 3D printing scaffolds | Promote mineralization<br>Hierarchical structure<br>Surface nanostructuring | High brittleness, low tensile strength, low impact resistance | [28,109] |
| | Bioactive glass | Fiber bonding<br>Wet spinning | Fiber-shaped scaffolds | Promote mineralization | Weak mechanical properties | [31,110] |
| dECM | dECM | Enzymatic digestion, solubilization | Hydrogel scaffolds | Natural osteogenesis inducing properties<br>Use of natural ECM | Laborious and time consuming processes | [36] |

Its intrinsic viscoelasticity is dependent on the frequency of its constituent D-mannuronic acid and L-guluronic acid blocks. Alginate is able to act as a drug delivery vessel due to its long release profile and possesses the interesting property of undergoing controlled in situ gelation, making it an interesting material for bioprinted scaffolds.[22] Abouzeid et al.[23] used biomimetic mineralization and alginate gelation properties to develop a biomimetic partial cross-linked alginate/2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) radical oxidized cellulose 3D printed nanofibril scaffold. The mineralization with HAp and the improved mechanical properties of this system, when compared to pure scaffolds of its components, suggests that this approach could be promising for BTE.[23]

The great versatility of chitosan makes it one of the most used materials in tissue engineering from nanoparticles to scaffolds. Chitosan has the particularity of having the same structure as glycosaminoglycans, a main component of the ECM. It also can support cell attachment, differentiation, and migration, making it an ideal base biomaterial for scaffold fabrication.[24] The use of chitosan to improve the biocompatibility of a system with nonorganic materials is shown by Pistone et al.[25] By incorporating HAp and magnetite in a chitosan matrix, they developed a hierarchical scaffold prepared by in situ precipitation for guided bone growth. Tests showed that the HAp and magnetite were homogeneously dispersed in the chitosan matrix. Moreover, it was shown that the incorporation of HAp and magnetite

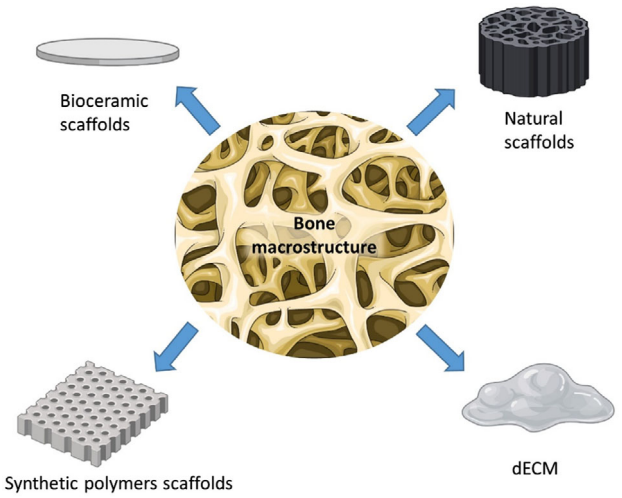


**Figure 2.** Bone structure and some of the most common approaches to mimic its hierarchical structure. Bioceramics, natural and synthetic polymers, and the dECM are the most used materials to try to recreate the bone architecture and specific composition.

provided the composite with overall improved biocompatibility and capacity to support osteoblastic cells' attachment and proliferation.[25]

The most widely used synthetic polymer in BTE is poly(lactic-*co*-glycolic acid) (PLGA). It is a very versatile material with multiple applications from the nano- and microrange. However, despite its degradation rate being easily tuned, a desirable property for scaffolds' design, its weak mechanical properties, and poor osteoconductivity limit cellular adhesion to the scaffolds.[26] For so, Kong et al.[27] used oxidized chondroitin sulfate (oCS) and collagen type I (oCS-Col I) coating to overcome the PLGA scaffolds' mechanical and osteoinductive shortcomings. The coating was performed by layer-by-layer deposition and further mineralized by nanohydroxyapatite (nHA). When compared to pure PLGA scaffolds, this system showed improved mechanical properties, as well as cell attachment, spreading, and proliferation. Furthermore, the biomimetic scaffolds promoted osteogenic differentiation of BMSCs, as shown by the upregulated expression levels of osteogenic markers (**Figure 3**).[27]

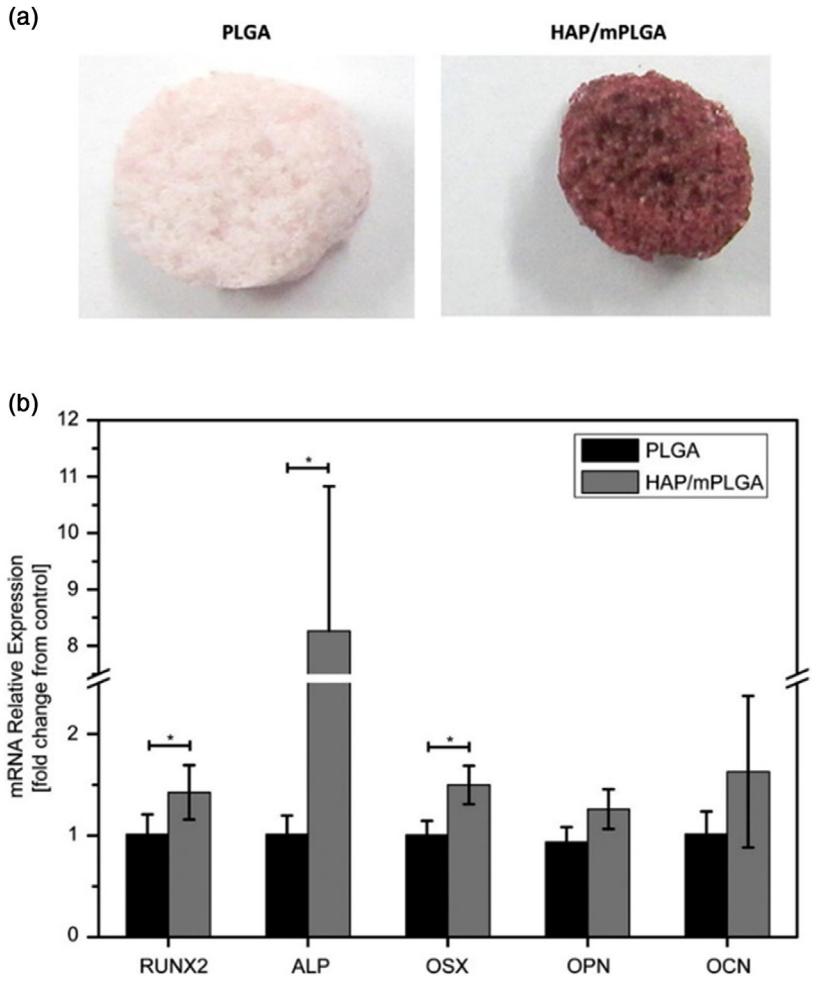


**Figure 3.** PLGA scaffolds coated with oCS-Col I. a) Pure PLGA scaffolds and PLGA coated with oCS-Col I multilayers after biomineralization (HAP/mPLGA). b) Quantification of osteogenic markers showing a visible increased expression in the HAP/mPLGA scaffolds in comparison with the pure PLGA scaffolds. Adapted with permission.[27] Copyright 2018, Wiley Periodicals, Inc.

### 2.2. Bioceramics

The use of bioceramics in bone scaffolds' fabrication has gained interest due to their strong mechanical properties and chemical similarity to native bone tissue. These biomaterials present biocompatibility, osteoconductivity, and a surface that absorbs osteoinductive factors, which facilitates osteogenic differentiation. The main disadvantage of ceramic materials is the high brittleness that can difficult degradation control.[12] A good example of its effect on osteogenic differentiation is shown in the work of Xu et al.[28] They developed 3D-plotted hierarchical β-tricalcium phosphate (TCP) scaffolds treated with dopamine-tris/HCl (TCP-DOPA-Tris) and dopamine-simulated body fluids solutions (SBF) (TCP-DOPA-SBF) to form apatite mineralization inducing structures (**Figure 4**). The nanostructured surface of these scaffolds improved osteogenesis as shown by bone-related gene expression and ALP activity tests performed on BMSCs, thus showing to be a viable tool for BTE.[28]

Bioactive glasses have the particularity to form reactive carbonated HAp when immerged into biological fluid, making it able to bond to mineralize bone tissue. Furthermore, ion release of bioactive glasses is known to stimulate gene transcription and expression of genes involved in osteoblast proliferation, ECM remodeling, and other important processes for bone homeostasis. The mentioned characteristics make bioactive glass an interesting material for bone regeneration application and an optimal base material for bone scaffold production.[29,30] For example, Fernandes et al.[31] developed a fiber bonding technique and wetspun porous poly-L-(lactic acid)-borosilicate bioactive glass scaffold for bone regeneration. The incorporation of bioactive glass resulted in a consistent release profile of inorganic species, producing calcium phosphate structures on the surface material. Additionally, in tests conducted with hASCs, the system promoted cell adhesion and proliferation, thus providing nontoxic structural support for cell growth.[31]

### 2.3. dECM

The natural characteristics of the ECM and its composition, alongside the difficulty to artificially replicate its properties

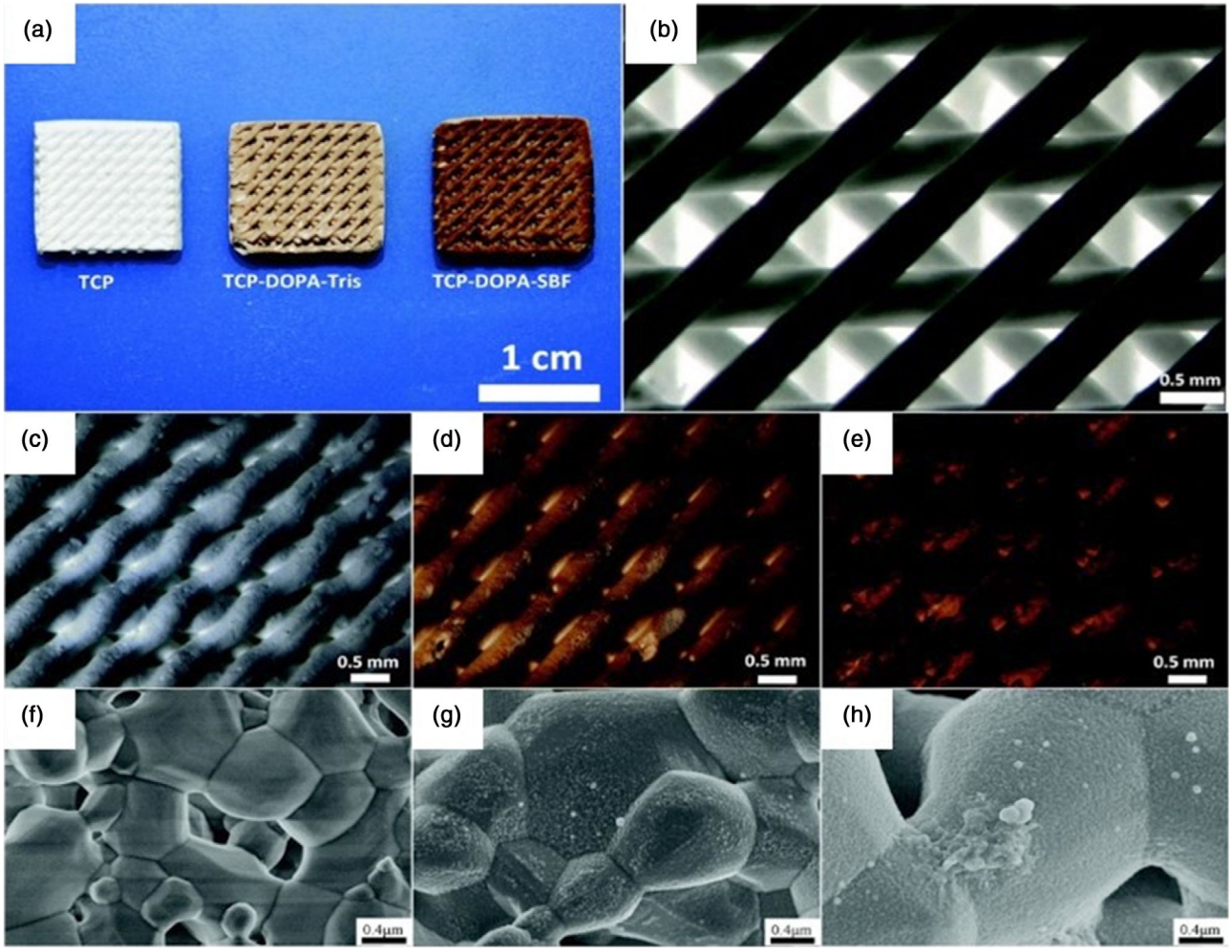


**Figure 4.** Photos of the 3D printed TCP, TCP-DOPA-Tris, and TCP-DOPA-SBF scaffolds. a) Overview of TCP, TCP-DOPA-Tris, and TCP-DOPA-SBF scaffolds b) in a 45–90–135° lay-down pattern. c,f) Visualization of macroporous and surface microstructures of TCP; d,g) TCP-DOPA-Tris; and e,h) TCP-DOPA-SBF. Reproduced with permission.[28] Copyright 2016, The Royal Society of Chemistry.

and complexity, make the dECM an ideal raw biomaterial for tissue engineering, far superior to any other engineering approach.[32] dECM can be obtained through decellularization of tissue and also cell-derived matrix (CDM). The decellularization process is of great importance for the further use and application of the ECM in tissue engineering. This process prevents the occurrence of immunological responses.[33] It is practically impossible to preserve entirely the natural properties of the ECM during decellularization. Every method will alter it in some form; hence, the goal of this process consists in the maximal removal of cellular material while altering as little as possible the structure and components of the ECM.[34] In the case of tissue-derived dECM, the harsh physical and chemical methods applied can lead to the extraction or denaturation of components of interest. Despite this, tissue-derived dECM retains most of its protein content and its complex 3D architecture, thus being an excellent scaffold material.[35] In the very interesting work of Alom et al.,[36] demineralized bone matrix (DBM) and decellularized bovine bone tissue (bECM) were used to prepare hydrogel scaffolds for osteogenic differentiation purposes. The bovine tissue was first demineralized in 0.5N HCl solution for 24 h, under agitation at 300 rpm and at room temperature. Afterward, the demineralized tissue was decellularized in 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid at 37 °C for 24 h. Hydrogels were then produced by enzymatic digestion, solubilization, and neutralization. Tests were performed with myoblasts and mouse primary calvarial cells, which were seeded on DBM, bECM, collagen type I, and tissue culture plastic in the presence and absence of an osteogenic medium (**Figure 5**). The expression of osteogenic markers showed a remarkable increase in expression in both DBM and mainly bECM when cultured in basal medium comparatively to tissue culture plastic and collagen type I. The results suggest that both demineralized and decellularized bone tissue are promising approaches for bioactive scaffolds for BTE.[36]

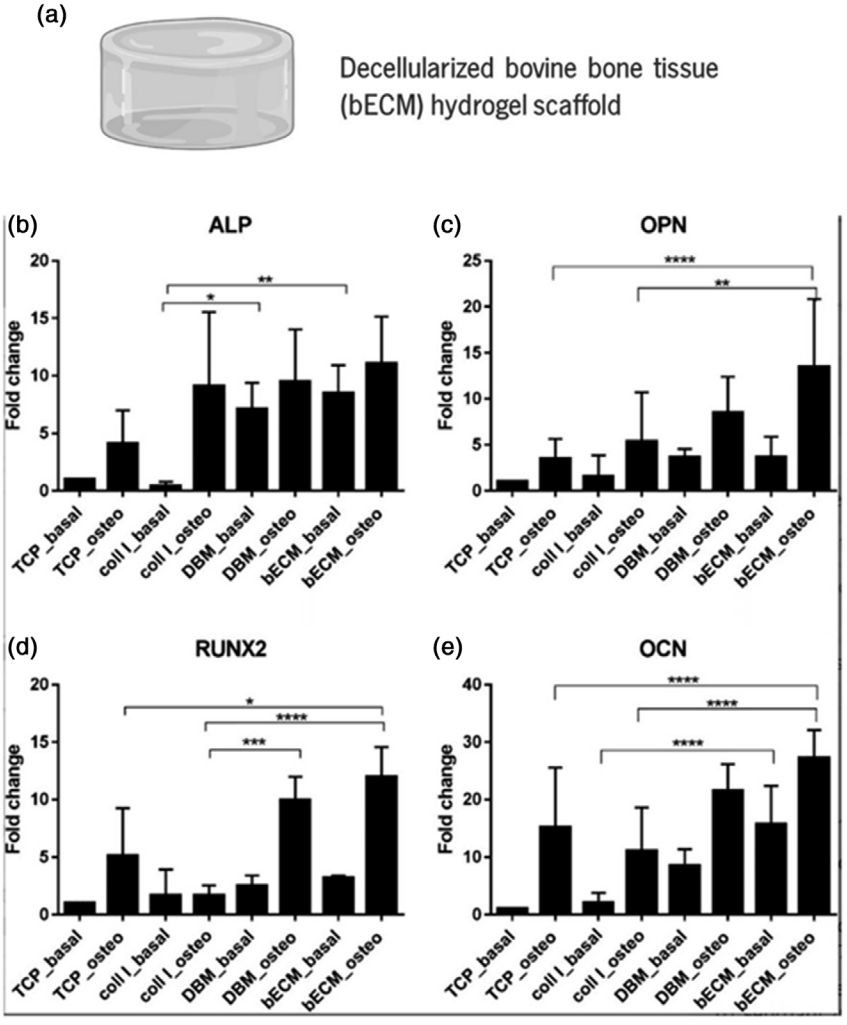


**Figure 5.** Decellularized bovine bone tissue (bECM) hydrogel scaffolds. a) Schematization of produced bECM hydrogel scaffolds. b–e) Expression of osteogenic markers in mouse primary calvarial cells seeded on bECM, DBM, collagen type I hydrogels, and tissue culture plastic cultured in basal and osteogenic medium. bECM evidenced the highest expression when cultured in a basal medium. Adapted with permission.[36] Copyright 2017, Wiley Periodicals, Inc.

Besides tissue-derived dECM, cell-derived matrices also present an interesting alternative to conventional tissue engineering approaches. Although these matrices present a lower complexity than tissue-derived matrices, CDM can be derived from autologous cells, allow for easier pathogen elimination, and can be created using patient-specific cells.[37] The use of a single cell type as a base for a cell culture model allows for a more precise analysis of its biological potential and also facilitates a high degree of customization. CDMs can be engineered by introducing soluble factors, proteins of interest, or coculturing different cell types before decellularization.[38] The use of dECM for scaffold fabrication, albeit its obvious advantages, is still in its early stages. However, this interesting technology will certainly be vastly explored in BTE, in a near future.

## 3. Micrometric Approaches

The recent advances in microfabrication processes have led to an increase of microscale approaches in biomedical engineering, like microscaffolds and microparticles-based approaches, as shown in **Figure 6**.

The ability to control the micrometric pore size, morphology, or distribution is a valuable tool for tissue engineering.[39] Furthermore, efficient drug delivery systems and mechanical reinforcement strategies are possible through this approach. **Table 2** summarizes the advanced fabrication techniques that can allow for a vast range of materials to be used in BTE.[40–47] The mimicking of the native tissue architecture and composition at a microscopic level is of high importance for the development of more complete and efficient tissue engineering approaches that more closely resemble natural bone.

### 3.1. Microscaffolds

The development of microscaffolds for BTE applications has gained attraction in recent years. A vast array of biomaterials and processing methods can be used to craft these devices for multiple applications. Gelatin, nHA, chitosan, calcium phosphate, or synthetic polymers like polycaprolactone (PCL) are the most used base biomaterials for these structures in BTE applications.[40–42,48]

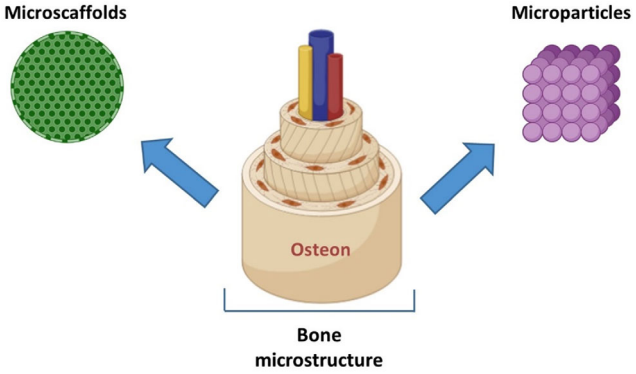


**Figure 6.** Bone microstructure and some of the most common approaches to mimic its hierarchical structure. Microscaffolds and microparticles are commonly used to imitate the microstructure or deliver bioactive molecules.

Gelatin is the denatured form of collagen and has a very similar composition to it, which makes gelatin a promising biomaterial for bone tissue strategies as shown in the work of Yin et al.[40] which combined gelatin and nHA to construct 3D microscaffolds within the pores of a porous titanium scaffold. Selective laser melting was used for the fabrication of porous titanium scaffolds and chemical cross-linking for microscaffolds production. This hybrid scaffold was tested for cell adhesion, proliferation, and differentiation with preosteoblasts cells. The results showed that this system presented good biocompatibility. Moreover, it was evidenced an increase of cell proliferation and enhanced cell adhesion, while gene expression analysis showed upregulation of osteogenic markers, leading to osteogenic differentiation. Furthermore, regenerated bone volume in vivo was also increased.[40] Showcasing the ability of chitosan, nHA, and gelatin to stimulate constituent components of natural bone, Wang et al.[41] tested an injectable microscaffold for the regeneration of subchondral bone knee lesions. These scaffolds were fabricated using a microstencil array chip, cryogelation, and lyophilization. In this study, the therapeutic potential was tested on an induced subchondral bone lesion, using rabbits as an animal model. The injected microscaffolds displayed optimal porosity, stiffness, swelling ratio, and favored cellular infiltration.[41] A different approach using microscaffolds was followed by Lin et al.[42] For this approach, the salt leaching and molding method was used to create calcium phosphate cement microscaffolds. The work aimed to combine the bioresorbable bone substitute calcium phosphate cement with osteoinductive hrBMP-2 adsorbed onto the microscaffold to achieve a more effective treatment for bone repair. The viability of this model was first tested on a rabbit distal femur defect model which yielded optimal bone regeneration 3 months after microscaffold implantation. In a pilot clinical trial, even compared to current clinical therapies, the implanted microscaffolds shortened the average fracture healing time by 0.5–2 months.[42]

In the case of synthetic materials, Totaro et al.[48] exploited PCL's excellent processability and mechanical properties to fabricate a porous PCL microscaffold functionalized with HAp nanoparticles for in vitro modular BTE. These microscaffolds were prepared via four-step thermally induced phase separation and tested for their capacity to support cell adhesion, growth, and osteogenic differentiation of human mesenchymal stem cells (hMSCs). Gene expression analysis results showed that the microscaffolds induced osteogenic differentiation even without the addition of exogenous osteogenic factors (**Figure 7**).[48]

### 3.2. Microparticles

Microparticles are particles in the 1–1000 μm diameter range.[49] They can be divided into microspheres and microcapsules according to their morphology.[50] In BTE, the use of microparticles for drug delivery purposes has been extensively explored. There is an ample group of biomaterials, from natural to synthetic, that can be used for microparticles production such as HAp, chitosan, gelatin, PGLA, or polylactide-poly(ethylene glycol) (PELA), among others.[43–47]

**Table 2.** Micrometric approaches used in BTE. nHA, nanohydroxyapatite; 3D, three-dimensional; PCL, polycaprolactone; PELA, polylactide-poly(ethylene glycol); PLGA, poly(lactic-*co*-glycolic acid).

| Category | Type of material | Fabrication methods | Scaffold strategies | Advantages of material | Disadvantages of materials | References |
|---|---|---|---|---|---|---|
| Microscaffolds | Gelatin | Chemical cross-linking | 3D microscaffolds with suitable pore size | Mimicry of natural bone elements | Low stability when exposed to higher temperatures | [40,111] |
| | | | | Suitable bone regeneration structure | | |
| | Chitosan | Microstencil array chip | Ready-to-use injectable microscaffolds | Suitable bone regeneration structure | Possesses high flexibility and therefore is not ideal for BTE applications. Low stability when exposed to higher temperatures | [41] |
| | Gelatin | | | Natural osteogenesis inducing properties | Low mechanical strength and slow degradation rate | |
| | nHA | | | | | |
| | Calcium phosphate | Salt leaching and molding method | Easy-to-operate pre-cured microscaffolds | Delivery of osteogenic differentiation promoters | High brittleness, low tensile strength, low impact resistance | [42,109] |
| | PCL | Phase separation process | Functionalized microscaffolds | Enhancement of mechanical properties | Low surface energy and resulting decreased cell adhesion | [48,112] |
| | | | | Natural osteogenesis inducing properties | | |
| Microparticles | PELA | Double emulsion/ solvent evaporation technique | Fused to scaffold | Delivery of osteogenic differentiation promoters | Poor osteoconductivity | [43] |
| | Chitosan | Emulsion-ionic cross-linking method | Incorporated into scaffolds | | Possesses high flexibility and therefore is not ideal for BTE applications | [44] |
| | PLGA | Freeze-drying technique | | | Poor mechanical properties and osteoconductivity | [26,47] |
| | | Water-oil-water emulsion | | | | |
| | Hap | Spray drying technique | Incorporated into scaffolds | Suitable bone differentiation and ECM synthesis promotion properties | Low mechanical strength and slow degradation rate | [45] |
| | Gelatin | Water-in-oil emulsion | Incorporated into scaffolds | Enhancement of mechanical properties | Low stability when exposed to higher temperatures | [46] |
| | | | | Delivery of osteogenic differentiation promoters | | |

As an interesting model for drug release, Ren et al.[43] studied the sequential release of BMP-2 and VEGF from PELA microcapsule-based scaffolds for bone defect treatment. Using the improved double emulsion/solvent evaporation technique, VEGF and BMP-2 were encapsulated in PELA microcapsules and fused to scaffolds using the dichloromethane vapor method. The results showed that the microcapsule-based scaffolds promote the differentiation of rat mesenchymal stem cells into osteoblasts as shown by the increased ALP expression, as well as quantification of extracellular signal-related kinase, a differentiation stimulator. The sequential release of BMP-2 and VEGF proved to be an interesting approach for the treatment of bone defects.[43] Similarly, to develop a system that allowed a controlled release of the growth factors and osteogenic proliferation, Sobhani et al.[44] incorporated BMP-2-loaded chitosan microspheres into polyphosphazene/calcium phosphate scaffolds. This process occurred by using calcium phosphate porous samples prepared through the sintering method and then composited with poly(dimethylaminoethanol)phosphazenes. The chitosan microspheres loaded with BMP-2 were incorporated in the poly(dimethylaminoethanol)phosphazenes. Moreover, they confirmed the nontoxic behavior of the scaffolds through cell adhesion and cell viability tests with rat osteosarcoma ROS17/2.8 cells, making this system a viable tool for BTE.[44] In a different approach, Li et al.[47] used chitosan due to its biocompatibility, biodegradability, and similarity to ECM. In this study, a porous chitosan/nHA composite scaffold was produced and improved with the osteogenic induction agent, simvastatin that was loaded into PLGA microspheres. The system was fabricated by combining freeze-drying techniques with the water–oil–water emulsion method. Drug kinetics showed that the release of the simvastatin happened in a sustained

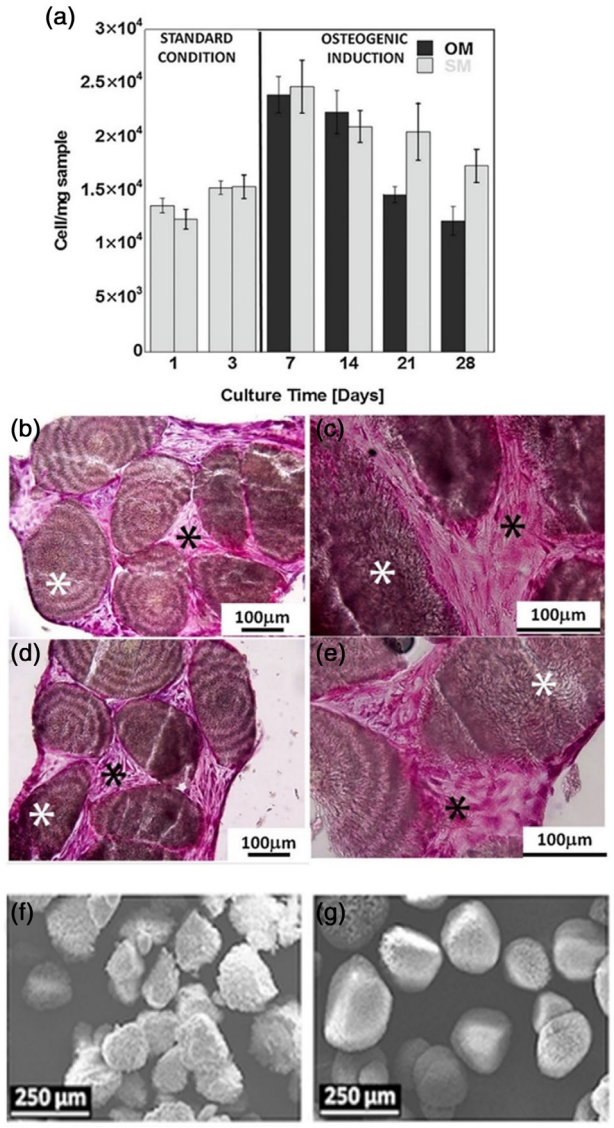


**Figure 7.** PCL microscaffolds with HAp nanoparticles for in vitro modular BTE. a) hMSCs proliferation on nanocomposite microscaffolds over the culture time, as measured by Alamar blue assay. A significant difference ($p < 0.05$) was detected between osteogenic medium (OM) and standard culture medium (SM) at 21 and 28 days. b–e) H&E staining after 28 days for both SM and OM showed complete integration of the cells by their ECM (white * indicates microscaffold and black * indicates de novo ECM). f) SEM image evidencing PCL structure; g) SEM image evidencing PCL-HA structure. Reproduced with permission.[48] Copyright 2015, John Wiley & Sons, Ltd.

manner, and the PLGA microspheres presented high encapsulation efficiency. Furthermore, in comparison to the chitosan/nHA composite scaffold without simvastatin, this system presented a better-suited microenvironment for BMSCs proliferation and increased osteogenic marker expression. Also, in a critical-sized calvarial defect rat model, a positive effect on bone repair was observed.[47]

In an interesting work, Cholas et al.[45] used microspheres to mechanically improve the scaffold system. They tried to produce biomimetic scaffolds using HAp mesoporous microspheres, which were prepared through spray drying precipitated HAp and then incorporated into collagen scaffolds (**Figure 8**). The porous nature of the collagen matrix allowed for the uptake of the microspheres and created a composite scaffold with superior compressive modulus when compared to pure collagen scaffolds. Despite the composite scaffolds being able to support cell growth, tests with preosteoblasts cells showed no effect on cells' proliferation. Interestingly, the microspheres affected the cells' morphology, as cells grown in the composite scaffold presented a rounded morphology similar to osteocytes while cells grown in collagen-only scaffolds presented an elongated shape.[45]

Another example showed the use of gelatin microspheres loaded with BMP-2 and incorporated into PLGA scaffolds to enhance osteogenesis.[46] Combining phase inversion and particulate leaching, a PLGA scaffold was prepared onto which water-in-oil emulsion obtained gelatin microspheres were incorporated via phase inversion. Tests were performed in vitro and on a defect radius of a rabbit after X-ray radiation in vivo. An enhancement of the mechanical properties of the PLGA scaffold was verified after the incorporation of the microspheres. Furthermore, in vitro results showed an improvement in proliferation and osteogenesis.[46]

## 4. Nanometric Approaches

The ever-greater incorporation of nanotechnology in tissue engineering approaches allowed for greater detail at the nanoscale necessary for reaching the next step in biomimetic tissue engineering (**Figure 9**).

In particular, the use of nanotechnology specifically tailored for the bone nanostructure, allowing for the development of more efficient drug delivery or mechanical reinforcement tools optimal for the bone tissue environment.[51] **Table 3** summarizes the several nanometric approaches comprising nanoparticles, nanocomposites, and nanosheets that can be used combined with top-down tissue engineering systems to more closely mimic the bone natural composition and its ECM.[52–71]

### 4.1. Nanoparticles

Nanoparticles are a valuable tool in BTE due to their versatile nature. Nanoparticles have shown promising results in promoting osteogenesis and osteointegration of other materials.[72,73] The major applications for nanoparticles in BTE are as vessels for delivering bioactive molecules and cell labeling. They are also commonly used in conjunction with scaffolds to enhance osteoconductive, osteoinductive, and mechanical properties for bone regeneration.[74] Nanoparticles used for BTE can be classified as degradable or nondegradable, according to their composing base material.[75] The selection of the base material highly depends on the desired end application of the nanoparticles.

#### *4.1.1. Degradable Nanoparticles*

Degradable nanoparticles can be prepared from various materials such as natural and synthetic polymers. Degradable nanoparticles are biodegradable and biocompatible, thus making

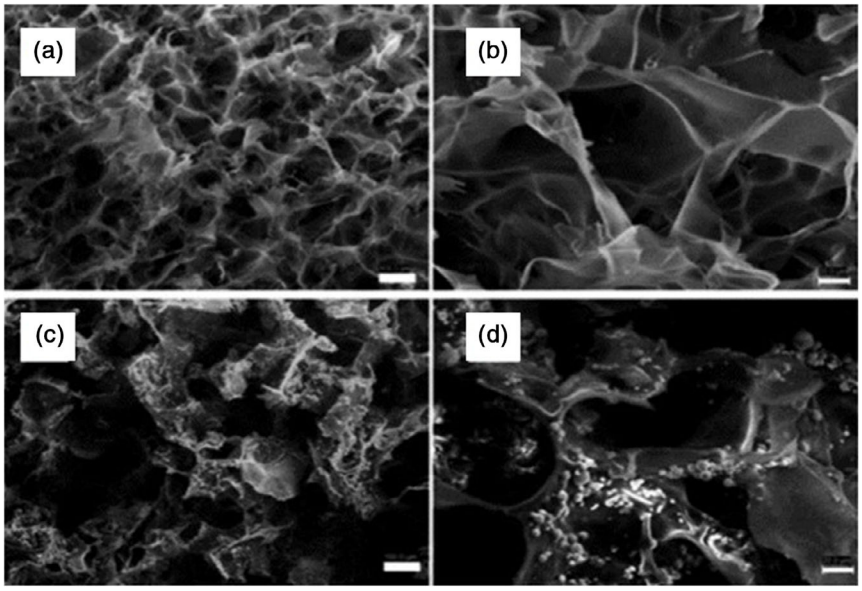


**Figure 8.** SEM images of collagen scaffolds. Comparison between a,b) pure collagen scaffolds and c,d) collagen/HAp mesoporous microspheres scaffolds. Scale bars: a,c) 100 µm, b,d) 20 µm. Reproduced with permission.[45] Copyright 2016. Elsevier B.V.

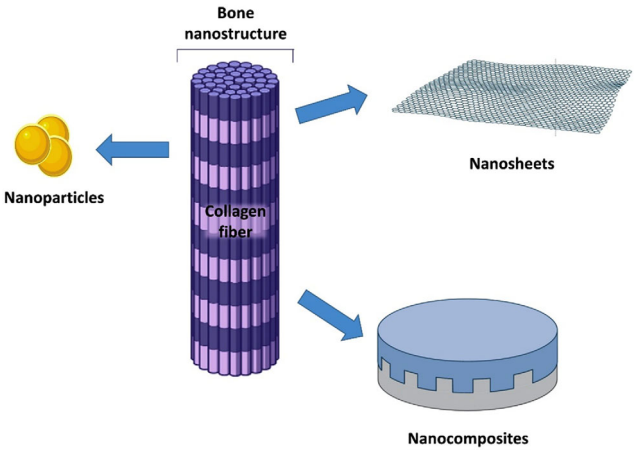


**Figure 9.** Bone nanostructure and some of the most common approaches at nanoscale to incorporate hierarchical elements. Nanoparticles, nanocomposites, and nanosheets are among the most studied approaches for delivery of biological active molecules or structural reinforcement.

them an interesting tool for drug delivery and scaffold assembly. In fact, they can be combined with bioactive components, which, when associated with hierarchical scaffolds, can more closely mimic the natural composition of the bone.[75] For example, bovine serum albumin (BSA) is a protein that has shown promising results in prolonging drug release due to its high half-life. The combination of high half-life with ease of administration and stability makes it an optimal drug carrier.[76] With this in mind, Xie et al.[53] developed BSA and silver nanoparticles (AgNPs) to be used as a delivery vessel for BMP-2. Nanoparticles were adsorbed to calcium phosphate mineralized graphene oxide/chitosan scaffolds with hierarchical structures. These scaffolds were fabricated by immersion of graphene oxide/chitosan scaffolds in supersaturated calcium and phosphate solution to achieve biomineralization. Furthermore, the scaffolds were immersed in the nanoparticle solution for adsorption. The BSA nanoparticles were produced using the desolvation method and stabilized with chitosan through electrostatic interaction. AgNPs were synthesized by reduction of silver nitrate. With this approach, they were able to not only enhance the osteoinductivity of the scaffolds but also enhance their antibacterial properties. In a different example, Han et al.[59] investigated chitosan-coated BSA nanoparticles and oxidized alginate deposited in a layer-by-layer manner on porous titanium scaffolds (**Figure 10**). Through sequential assembling of chitosan-coated BSA nanoparticles and negatively charged oxide alginate on the titanium scaffolds, BMP2 dropped and absorbed on the layer-by-layer film. The chitosan-coated BSA nanoparticles were synthesized via desolvation.

Using this strategy, they were able to produce nanostructured scaffolds with improved surface biocompatibility, functionality, and loaded with growth factors and antibacterial agents. This modification enhanced ectopic bone formation in vivo, improved cell adhesion, cell proliferation, and induced osteogenic differentiation of bone marrow stromal cells in vitro as confirmed by ALP activity assay.[59]

Another material with good biocompatibility is SF, a hydrophobic structural protein composed of an equimolar ratio of a heavy hydrophobic chain and a light relatively hydrophilic chain of protein, most commonly obtained from *Bombyx mori*.[77,78] The ability to use several processing techniques to obtain a vast array of morphologies and scaffold types, from nanoparticles to hydrogels, is another great advantage of SF.[19] More specifically, the SF structure similarity to the native structure of collagen type I combined with its excellent mechanical properties, slow degradation rate, and high morphology control makes it an extremely

**Table 3.** Nanometric approaches used in BTE. BSA, bovine serum albumin; PLGA, poly(lactic-*co*-glycolic acid); Hap, hydroxyapatite; ECM, extracellular matrix; PLLA, poly (L-lactic acid).

| Category | Type of material | Fabrication method | Scaffold strategies | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|
| Nanoparticles | BSA | Desolvation method | Adsorbed to scaffolds | Delivery of osteogenic differentiation promoters | Cytotoxicity | [53,113] |
| | Silver | | | | Reduced biocompatibility | |
| | | | | | 1D applicability | |
| | PLGA | Co-axial electrospraying method | Mixed within the material solution | | Poor mechanical properties and osteoconductivity | [54] |
| | Silica | Sol–gel synthesis | Deposition on scaffold surface | | Lack of organic functional groups can difficult incorporation in other scaffolds | [55,113] |
| | | | | | Cytotoxicity | |
| | Dendrimers | Precipitation and freeze drying | Dispersed in cell media | | Can cause a decrease in cell viability | [56,113] |
| | Silver | Chemical reduction | Mixed within the material solution; adsorbed to scaffolds | Enhancement of mechanical properties | Cytotoxicity | [57,113] |
| | | | | | Reduced biocompatibility | |
| | Silver | Aqueous precipitation reaction | Mixed within the material solution | Enhancement of mechanical properties. | Weak osteogenic capacity | [52,113] |
| | Silk fibroin | | | Mimicking of natural bone and/or ECM nanostructure | Degradation products impact cell activity | |
| | | | | | Cytotoxicity | |
| | | | | | Reduced biocompatibility | |
| | Carbon | Coprecipitation method | | Enhancement of mechanical properties | Cytotoxicity | [58,113] |
| | Magnetite | | | Therapeutic options through photothermal activity | High chemical stability can difficult covalent functionalization | |
| | BSA | Desolvation method | Layer-by-layer deposition on surface | Improvement of surface biocompatibility and biofunctionality | 1D applicability | [59] |
| | | | | Mimicking of natural bone and/or ECM nanostructure | | |
| | Nanobioglass | Facile one-pot ultrasound/sol–gel synthesis | Deposition on scaffold surface | Mimicking of natural bone and/or ECM nanostructure | Weak mechanical properties | [60,114] |
| | Gold | Surface growing | Deposition on scaffold surface | Natural osteogenesis inducing properties | Reduced biocompatibility | [61,113] |
| | | | | | Cytotoxicity | |
| | Hap | Wet-precipitation method | Mixed within the material solution; | | Low mechanical strength and slow degradation rate | [62] |
| | Gold | Reduction method | Mixed within the material solution | Cell proliferation stimulation by direct current | Reduced biocompatibility | [63] |
| | Titanium | Chemical reduction | | | Cytotoxicity | |
| | Platinum | | | | Chemically inert | |
| | Iron oxide | Coprecipitation method | | Creation of electromagnetic field for enhancement of regenerative process | Cytotoxicity | |
| | Carbon | | | | | [64] |

**Table 3.** Continued.

| Category | Type of material | Fabrication method | Scaffold strategies | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|
| Nanocomposites | Clay-polyurethane | Solvent evaporation–dissolution technique | Used as scaffold material | Delivery of osteogenic differentiation promoters | Weak mechanical properties | [65,115] |
| | | | | Enhancement of mechanical properties | Low osteoinductivity | |
| | Hap | One-step solvothermal method | Incorporated into scaffold matrix | Enhancement of mechanical properties | Low mechanical strength and slow degradation rate | [66,116] |
| | | | | Mimicry of natural bone elements | | |
| | Nylon 6 | Sol–gel method | | | Low osteoinductivity | |
| | Baghdadite | | | | Brittleness and poor mechanical stability | [67] |
| | | | | Hierarchical structure | | |
| | Palladium oxide–titanium dioxide | Sacrificial template | | Enhancement of mechanical properties | Reduced biocompatibility | [68,113] |
| | | | | | Cytotoxicity | |
| Nanosheets | Graphene oxide | Spin coating and peeling techniques | Conjugated with other material and surface adsorbed | Delivery of osteogenic differentiation promoters | Challenging to deposit on certain materials | [69,117] |
| | Black phosphorous | | | Natural osteogenesis inducing properties | Lack of stability | |
| | PLLA | Oxidation and exfoliation | Sandwich-type approach | | Hydrophobic nature can difficult active molecule adsorption | [70,118] |
| | Graphene oxide | Modified Hummers method | Deposited on scaffold surface | Hierarchical structure | Challenging to deposit on certain materials | [71] |

versatile material for BTE applications.[77] Huang et al.[52] took advantage of the negative charge repulsion caused by the silk shell to prevent nanoparticle aggregation. In this work, aqueous precipitation reaction synthesized HAp/SF core–shell nanoparticles were combined with ECM mimetic porous SF scaffolds produced through freeze-drying. This resulted in scaffolds with homogenous nanoparticle distribution, higher HAp content by 40%, higher ALP activity, and higher calcium deposition, achieving better growth and osteogenic differentiation.[52]

In the special case of synthetic polymers, such as PLGA, they have not only the advantage of being cost-effective, easy to produce, and biocompatible, but also that the degradation profile can easily be tuned to achieve optimal release profiles. Even through PLGA hydrolytic degradation, which produces acidic products, can be a problem.[79] Evidencing the mentioned degradation properties, Zhou et al.[54] used PLGA nanoparticles to encapsulate transforming growth factor-beta (TGF-β1). The goal of this study was to develop a 3D printed scaffold with a hierarchical structure for osteochondral regeneration. The PLGA nanoparticles with encapsulated TGF-β1 were produced via the coaxial electrospraying method. Then, using 3D printing technology, gelatin methacrylate (GelMA) and polyethylene (glycol) diacrylate (PEGDA) were used as primary ink in a layer-by-layer manner deposition using a stereolithography-based 3D printer. By using PLGA nanoparticles, the release of the growth factors was modulated onto BMSCs, emulating the bone composition. This interesting scaffold design successfully upregulated the expression of osteogenesis-associated genes. The results evidenced the osteogenic differentiation potential of this system, which was further confirmed by histological staining of calcium deposits with Alizarin red.[54]

#### 4.1.2. Nondegradable Nanoparticles

Nondegradable nanoparticles are usually composed of a wide range of materials like ceramics, metals, among others, that can be used to produce several constructs in a wide spectrum of sizes and shapes.[75] These nanoparticles, although mostly displaying some levels of toxicity, can make use of the inherent properties of their raw material allowing them, for example, to create magnetic fields that can allow envisioning the use of magnetic nanoparticles.[80,81] Nondegradable nanoparticles have a vast variety of applications such as drug delivery, imaging, and reinforcement of the mechanical structure of scaffolds.[82]

One example of widely used nondegradable metal nanoparticles in BTE is the gold nanoparticles (AuNPs). AuNPs possess good biocompatibility, low toxicity, and tunable stability, making them a versatile tool for drug delivery, imaging, and diagnosis among other applications.[83] Moreover, AuNPs have been shown

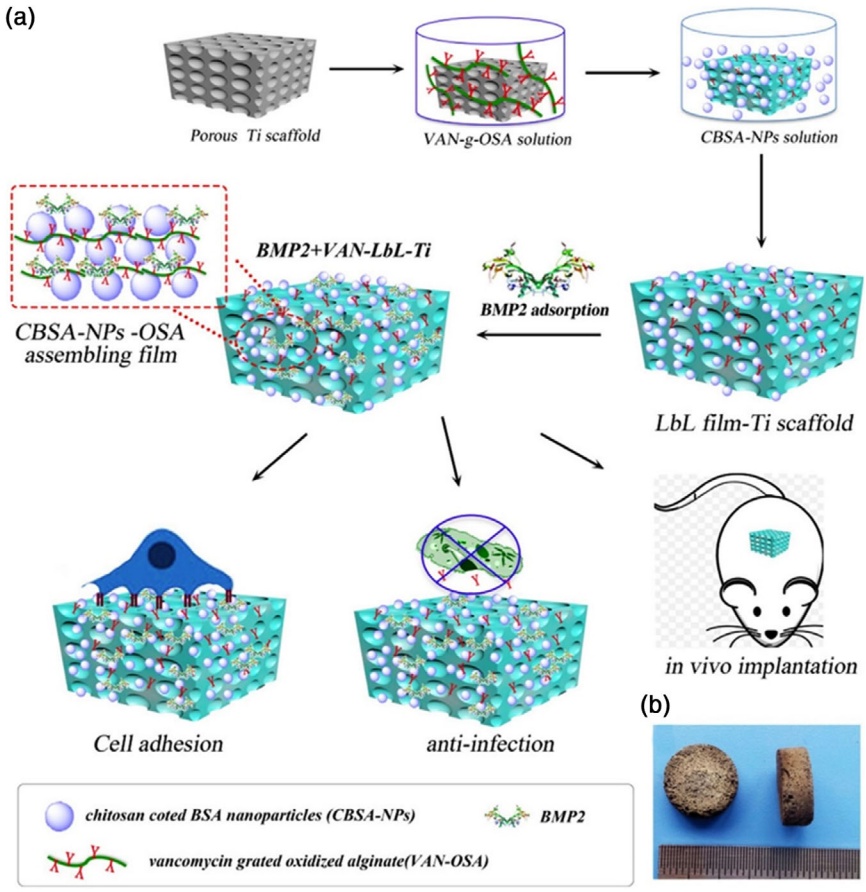


**Figure 10.** Chitosan-BSA nanoparticles loaded–oxidized alginate titanium scaffolds. a) Schematics of chitosan-coated BSA nanoparticles and oxidized alginate deposited in a layer-by-layer manner on porous titanium scaffolds for bone regeneration and anti-infection as an example of nanoparticles used to improve bone regenerative scaffold systems. b) Titanium scaffolds before modification. Adapted with permission.[59] Copyright 2017, Wiley Periodicals, Inc.

to promote osteogenic differentiation[84] as shown by Lee et al.[61] They took advantage of the AuNPs osteoinductivity to develop a 3D printed PCL scaffold coated with AuNPs grown on a polydopamine coating. PCL scaffold was produced using a rapid prototyping 3D printing system and evenly coated with polydopamine. The coating acts as a reductant of gold ions forming AuNPs. The use of polydopamine coating led to an increase in AuNPs' growth on the scaffolds in comparison with standard PCL scaffolds. The combination of a higher nanoparticle growth due to the polydopamine coating led to great results in osteogenic activity as shown by the increase in ALP activity, exhibiting excellent new bone formation in in vivo tests.[61] Another example is the silver nanoparticles (AgNPs). The antibacterial properties of Ag make it very appealing for tissue engineering approaches.[85] For example, these properties were used by Correia et al.[57] to develop 3D printed TCP/sodium alginate scaffolds with bactericidal activity. TCP/sodium alginate scaffolds were produced by rapid prototyping 3D printing and functionalized with AgNPs by adsorption and direct incorporation into the composite mixture. The direct incorporation of AgNPs produced a system suitable for BTE with biocompatibility, and appropriate mechanical properties. Both functionalization methods showed great antibacterial activity.[57] Other metals like titanium and platinum, although rarer, are also used in BTE for nanoparticle synthesis. These materials allow for controlled sized range, functionalization, topography, and use as contrast agents.[86] Platinum has been shown to exhibit an optimal catalytic ability and anti-inflammatory ability. This is due to its capacity to impair the downstream pathways leading to inflammation,[87] while titanium implants with rough topography and free energy increased cell adhesion and subsequent bone formation.[88] In the very interesting work of Radwan-Praglowska et al.,[63] three different metal nanoparticles were used (platinum, gold, and titanium dioxide (TiO2)) to dope 3D hierarchical, nanostructured chitosan/polylactic acid (PLA)/HAp scaffolds for guided BTE. These scaffolds were prepared by placing a nanofibrous 3D PLA mat onto a chitosan aerogel followed by lyophilization and embedding of Au, TiO2, or platinum nanoparticles at 1%. In fact, the conductivity characteristic of the nanoparticles enabled the stimulation of cell proliferation by inducing an electric current. Moreover, they exhibited antibacterial activity, which improved the mechanical properties of the scaffolds. In this study, comparing the three nanoparticles tested, the $TiO_2$ nanoparticles samples displayed the highest bioactivity in contact with cells. It was also verified

that scaffolds containing AuNPs showed the highest increase in biomineralization.[63]

The use of magnetic nanoparticles in BTE is also a promising approach. In fact, it is known that magnetic fields can improve the integration of implants and the mineral density of newly formed bone and promote faster defect healing.[81] These features associated with the possibility of magnetic nanoparticles serve as drug delivery and gene transfection vessels, which have prompt scientists to explore them extensively in recent years.[89] A combination of these properties was shown by Saber-Samandari et al.[90] They used magnetic nanoparticles as photothermal conversion agents to develop bifunctional nanocomposite scaffolds for photothermal therapy and tissue engineering. The entrapping of carboxyl-functionalized multiwalled carbon nanotubes and embedding of magnetic iron oxide nanoparticles into the porous matrix of the scaffold resulted in the increased adsorption of BSA on the surface. Additionally, they also observed that the presence of magnetic nanoparticles improved the biocompatibility, as confirmed using an osteoblastic cell line, and the compressive strength values of the scaffolds.[90]

Another interesting approach is the use of carbon for nanoparticle development. The unique and versatile properties of carbon have made it an ideal candidate for the development of new BTE strategies. Carbon can be processed in several different dimensions, from 3D graphite, 2D graphene, 1D carbon nanotubes to 0D carbon dots or carbon nanodiamonds.[91] The most used iteration of carbon nanoparticles is carbon nanotubes which can be single-walled carbon nanotubes (SWCNTs) or multiwalled carbon nanotubes (MWCNTs). These possess good strength, elasticity, and fatigue resistance and can be used to improve the overall mechanical properties of constructs. Their high affinity for cell binding proteins can promote stem cell differentiation and their electrical conductivity can be controlled by adjusting size and diameter.[92,93] Additionally, the carbon nanotubes' unique electrical conductivity has also gained increasing interest in BTE. Electrical stimulation can accelerate bone formation, regeneration, ECM protein synthesis, and enhance osteogenic markers expression.[94] Another important advantage of carbon nanotubes is that their micrometer length and nanometer diameter resembles constituents of ECM of connective tissue such as collagen fibrils.[64] With this in mind, Święt ek et al.[64] used hydroxylated MWCNTs in combination with magnetic iron oxide nanoparticles incorporated into a porous PCL matrix to create a multifunctional system that enables multiway cell stimulation. These hybrid nanoparticles were synthesized by coprecipitation, and the solvent casting/porogen leaching method was used to fabricate the scaffolds. This preliminary research developed with an osteoblastic cell line showed that the carbon nanotubes had a positive effect on cell adhesion.[64]

Bioceramics are also commonly used in BTE, silica, HAp, calcium phosphate, and bioglasses are some of the most used bioceramics. On one hand, silica nanoparticles have shown to be very interesting for tissue engineering due to their excellent bioactivity, low toxicity, and bone tissue development properties when in contact with physiological fluids[95] as shown by Qiu et al.[55] In this study, they used aminated mesoporous silica nanoparticles (MSNs-$NH_2$) as drug delivery vessels for dexamethasone onto poly (L-lactic acid) (PLLA)/PCL composite scaffolds for BTE. PLLA/PCL composite scaffolds were fabricated via thermally induced phase separation on which drug-loaded silica nanoparticles were placed with electrophoretic deposition. These nanoparticles were produced using sol–gel synthesis. The results obtained on BMSCs showed a sustained release of dexamethasone and higher ALP activity and collagen content after 21 days when compared to scaffolds without dexamethasone-loaded silica nanoparticles (**Figure 11**).[55]

On the other hand, HAp is an abundant element in the natural composition of bone tissue, reinforcing the organic part of the bones' hierarchical structure.[96] For so, to better mimic bone tissue, Guillaume et al.[62] used HAp nanoparticles to dope composite polymer scaffolds to promote bone repair. Photo-cross-linkable poly(trimethylene carbonate) (PTMC) was prepared with different contents of HAp nanoparticles; these were obtained using the wet precipitation method. For composite scaffolds fabrication stereolithography was used, obtaining

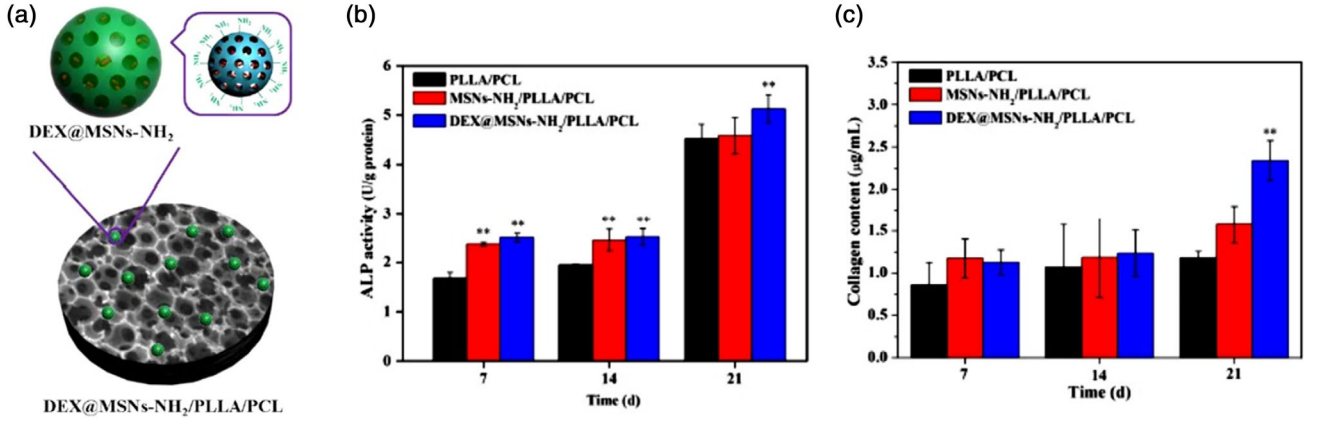


**Figure 11.** Dexamethasone-loaded MSNs-$NH_2$ PLAA/PCL scaffolds for BTE applications. a) Schematic representation of dexamethasone-loaded MSNs-$NH_2$ PLAA/PCL scaffolds (DEX@MSNs-NH2 PLAA/PCL) illustrating the different interactions between the MSNs-$NH_2$, dexamethasone, and the scaffold. b) In vitro ALP activity, and c) collagen content of BMSCs cultured on the tested scaffolds showed evident differences between DEX@MSNs-$NH_2$ PLAA/PCL and scaffolds without dexamethasone (PLLA/PCL and MSNs-$NH_2$ PLAA/PCL), demonstrating the drug delivery capacity of MSNs-$NH_2$. Adapted with permission.[55] Copyright 2016, American Chemical Society.

PTMC/HAp scaffolds. The surface-enrichment with HAp allowed a microscale distribution at the surface of the structures, and bone formation was obtained even at low concentrations of HAp.[62] In fact, calcium phosphates are osteoconductive, osteoinductive, and have the ability to be resorbed by the cells making them a great tool for drug delivery,[97] as shown in the very interesting work of Chen et al.[98] They developed composite scaffolds of collagen and dexamethasone-loaded biphasic calcium phosphate nanoparticles to achieve a sustainable release of dexamethasone, calcium, and phosphorous ions. The nanoparticles were produced using the coprecipitation method, loaded with dexamethasone, and hybridized with collagen. The dexamethasone-loaded scaffolds were fabricated using preprepared ice crystals as porogen material. The obtained nanoparticle/collagen/ice solution was poured into silicon frames placed on copper plates, and then the frames were frozen and freeze-dried. The homogeneous distribution of nanoparticles enhanced the mechanical properties as well as the roughness of the scaffolds. Tests performed with hMSCs showed that the system displayed good biocompatibility and promoted osteogenic differentiation. In fact, an increase in ALP activity and osteogenic gene expression, as well as new bone tissue regeneration were also evidenced.[98]

Bioglass has emerged as an interesting ceramic tool to be used for hierarchical scaffold production. Furthermore, nanobioglass has shown potential for osteogenic differentiation; because it is a calcium-silicate-based ceramic it is able to initiate the formation of carbonated HAp that mimics the natural bones ECM[99] Exploring this potential, El-Fiqi et al.[60] developed a biomimetic silanized mesoporous nanobioglass/collagen scaffold. This system was biomimetically mineralized to enable the growth of nHA crystals and hence more closely resemble natural mineralization. The nHA/collagen porous scaffolds were prepared through biomimetic mineralization of mesoporous nanobioglass/collagen hybrid porous scaffolds. Facile one-pot ultrasound sol–gel synthesis was used for nanobioglass synthesis. These scaffolds exhibited excellent in vivo osteogenic potential in tests conducted in rat calvarial bone defects compared to nonmineralized scaffolds. The improvement of physicochemical and mechanical properties was also verified as mineralized scaffolds presented higher density and stiffness.[60]

Finally, one of the most used drug-delivering tools is dendrimers; these nanoparticles possess optimal physical and chemical properties that make them suitable for drug delivery tools. They possess low cytotoxicity, high biocompatibility, and their surface charge can be manipulated to interact with targeted biosystems. Dendrimers have a well-defined nanosized structure that allows them to cross cell membranes without premature elimination from the body and can be internalized by several cell types making it a very appealing strategy.[100,101] For example, Oliveira et al.[56] used dendrimers' drug carrier capacity to fabricate dexamethasone-loaded carboxymethyl chitosan/poly(amidoamine) dendrimer nanoparticles. These dendrimers with a 3D system of both HAp scaffolds and starch–PCL were studied for osteogenic differentiation on rat bone marrow stromal cells. In brief, carboxymethyl chitosan and poly(amidoamine) dendrimers were mixed with dexamethasone in an aqueous solution, precipitated, submitted to dialysis, and freeze-dried. Afterward, the resulting dendrimers were cultured onto the macroporous scaffolds. Although quite similar, the results showed an increase in osteoblast differentiation and enhancement of osteogenesis in the HAp scaffolds system when compared to starch–PCL scaffolds. An increase in ALP activity and mineralization of the ECM was also verified. The observed osteogenic differentiation showed that these dendrimers may be used as suitable intracellular nanocarriers for bioactive factors.[56]

### 4.2. Nanocomposites

Nanocomposite biomaterials are a recent class of materials that can be defined as multiphase solid material in which one of the phases has one, two, or three dimensions less than 100 nm.[102] There are different types of nanocomposites: organic–inorganic, inorganic–inorganic, and bioinorganic nanocomposites, classified according to their composition.[102] Nanocomposites can be engineered to mimic the hierarchical nanostructure of native tissue. They are a versatile tool with multiple applications such as drug delivery, cancer therapy, imaging, clinical sensing, and mechanical reinforcement.[102,103] More specifically, studies have demonstrated nanocomposite capacity to promote cell adhesion, cell proliferation, and osteogenic differentiation, making them an interesting tool for BTE.[104,105] Exemplifying the tissue engineering potential of such a tool, Norouz et al.[65] developed a new nanocomposite scaffold composed of polyurethane and surface-modified clay nanoplates for osteogenic differentiation. First, clay nanoplates were surface modified with phosphoric acid and calcium hydroxide, and then the clay nanoplates were combined with polyurethane by a solvent evaporation–dissolution technique forming the nanocomposites. ALP activity assay and gene expression analysis showed a significant enhancement of human adipose-derived mesenchymal stem cells (hADSCs) proliferation and osteogenic differentiation. Results of this study suggest that surface-modified clay nanoplates mediate osteoinductive and osteoconductive responses of hADSCs.[65] Envisioning the improvement of scaffolds' mechanical properties, Sun et al.[66] developed a novel nanocomposite comprising ultralong HAp nanowires decorated with zinc-containing nanoparticles to mechanically reinforce and promote osteogenesis. Nanoparticles were produced using the one-step solvothermal method and the nanocomposite was incorporated into a chitosan matrix produced through freeze-drying. In tests performed with rat BMSCs, osteogenic markers expression analysis revealed enhanced osteogenic differentiation. Micro-CT analysis showed that the developed nanocomposite favored in vivo bone regeneration when compared to pure porous chitosan scaffolds.[66] With the same goal in mind, Abbasian et al.[67] introduced a biomimetic 3D scaffold with a hierarchical microstructure achieved by combining nylon 6, baghdadite nanopowder (**Figure 12**), and a sacrificial cuttlefish bone template. The scaffolds were fabricated using impregnation of the N6-baghdadite solution onto a sacrificial cuttlefish bone template in vacuum. The use of the baghdadite nanopowder showed an improvement in the overall mechanical properties and bioactivity of the system.

Tests using an osteoblastic cell line showed that nanocomposite scaffolds promoted cell attachment, spreading, and proliferation.[67] In the case of the work of Vedhanayagam et al.,[68] they developed a method to mechanically reinforce collagen scaffolds

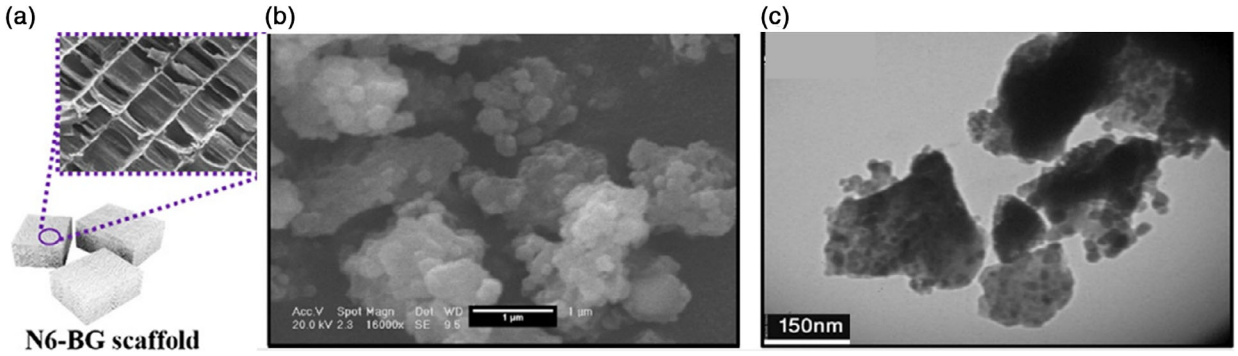


**Figure 12.** Nylon 6-Baghdadite nanocomposite scaffold. a) Biomimetic nylon 6-Baghdadite nanocomposite scaffold and its microstructure. b) SEM and c) TEM images of baghdadite nanopowder. Adapted with permission.[67] Copyright 2019, Elsevier B.V.

without affecting their conformation or biocompatibility. Poly(methyl) methacrylate grafted collagen scaffolds were reinforced with palladium oxide–titanium dioxide nanocomposites that were produced using the sol–gel method. When compared to pure collagen scaffolds, the nanocomposite enhanced scaffolds showed higher thermal stability, mechanical strength, and enhanced osteogenic differentiation of an osteogenic cell line. These results were confirmed by ALP activity assay and Alizarin Red staining of calcium deposits.[68]

### 4.3. Nanosheets

Another recent nanometric approach studied in BTE is the use of nanosheets. These 2D materials have gained great interest in recent years due to their ability to alter surface properties and thus improve the biocompatibility and cell affinity of scaffolds.[69] Nanosheets can be used in combination can be used in combination with other biomaterials or as standalone approaches to enhance the mechanical properties of bone scaffolds but also for drug delivery. The most used materials for nanosheets application in bone scaffolds are graphene oxide, synthetic polymers like PLLA or PCL, and black phosphorous.[69–71]

One example of using PLLA nanosheets is described at Murahashi et al.[70] A sustainable release drug carrier was developed using multilayered PLLA nanosheets loaded with fibroblast growth factor-2 (rhFGF-2) for bone regeneration, as schematically described in **Figure 13**. Nanosheets were fabricated using spin coating and peeling techniques. In performed tests on critical-sized mouse femoral defects, the multilayered nanosheets induced more efficient bone regeneration when compared to rhFGF-2-loaded gelatin hydrogels. Even after 4 weeks, trilayer nanosheets continued to show growth factor release, along with osteoblast differentiation associated with fibroblast growth receptor 1 activation.[70]

Considering the graphite oxide nanosheets, their unique structure and physicochemical properties prompted extensive research for biomedical applications. In fact, its high surface area, biocompatibility, biodegradability, and rich oxygen-containing functional groups make them an ideal material for scaffold fabrication.[106] Taking advantage of graphene oxide's physicochemical properties, Han et al.[71] used graphene oxide

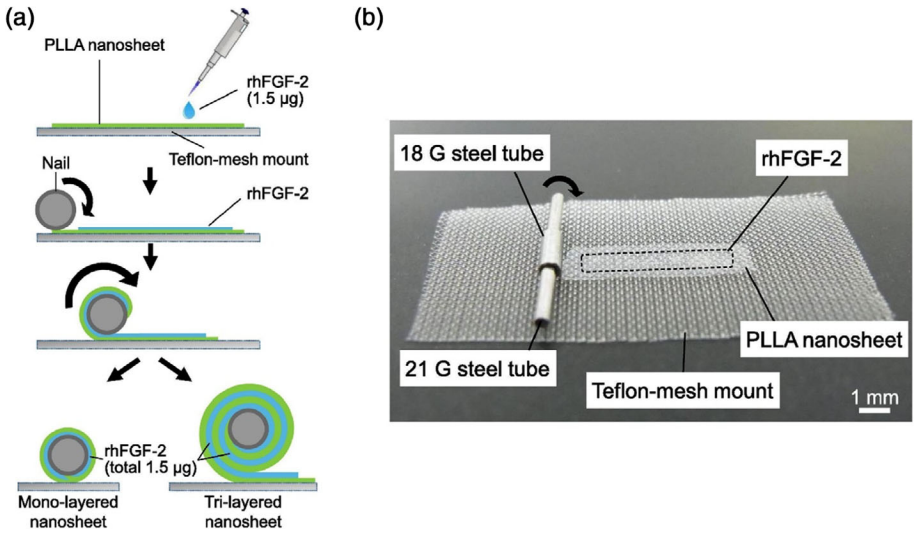


**Figure 13.** Multilayered PLLA nanosheets. a) Schematic representation of the process for production of multilayered PLLA nanosheets loaded with rhFGF-2. b) Completely assembled system. Adapted with permission.[70] Copyright 2018, Acta Materialia Inc. Published by Elsevier Ltd.

nanosheets to functionalize inert titanium scaffolds, making them chemically active. Additionally, BMP-2 and antibiotic (vancomycin)-loaded gelatin microspheres were immobilized in the graphene oxide nanosheets by electrostatic interaction providing an effective drug delivery system. Graphene oxide/titanium scaffolds were produced using an immersing method under vacuum filtration and gelatin microspheres were then immobilized on the surface of the scaffold. The results of the ALP activity of BMSCs along with in vivo ectopic bone regeneration showed that this system was efficient in inducing osteogenic differentiation and bone regeneration.[71]

Finally, in the case of black phosphorous, it has been shown that this allotrope of phosphorous exhibits fantastic properties for biomedicine such as excellent optical and mechanical properties, electrical conductivity, good biocompatibility, and good biodegradation.[107] Furthermore, the degradation product of black phosphorous is phosphate anions, a component of bone tissue and osteoblast differentiation facilitator.[108] This makes black phosphorous an ideal nanomaterial for BTE, as shown in the interesting work of Liu et al.[69] They used black phosphorous nanosheets and graphene oxide nanosheets for surface coating of 3D stereolitography printed scaffolds with the goal to enhance cell proliferation and osteogenesis. The graphene oxide nanosheets' high surface area enabled high protein adsorption and cell adhesion on the scaffolds. Tests performed on preosteoblast cells showed the development of cellular filaments around black phosphorous nanosheets, elongated cell shape, and the release of calcium phosphate with the slight enhancement of biomineralization. Furthermore, biomineralization and cellular osteogenic markers values suggest that the combined use of black phosphorous and graphene oxide nanosheets was more efficient in stimulating cell proliferation and osteogenesis than the nanosheets individually.[69]

## 5. Conclusion and Future Directions

The development of biomimetic scaffolds that more closely resemble natural bone tissue remains a challenge. In order to create an ideal template for bone regeneration, the mimicking of bone tissue and ECM in both its nano- and macroenvironment is key. This presents an especially difficult task due to the complexity of the bone environment. Evermore precise fabrication of proper structure, organization, and bioactive constitution in scaffolds is needed to explore the full potential of BTE as a viable alternative to autografts. Novel processing and fabrication technologies are being used and studied to further the advances in the field. The incorporation and combination of biomaterials that are able to structurally mimic the native tissue and its nano/microscale chemical and physical properties is a step forward to develop better hierarchical scaffolds and a step closer to provide better clinical solutions. 3D printing technology seems to be at the forefront of fabrication strategies that allow a wide and precise control over the properties of the construct, and its use for BTE purposes is a growing trend. The recent nature of this technology could suggest that progress in the 3D printing area could improve scaffold production for tissue engineering. From the analysis of the literature, it is clear that scaffolds must have an optimal architecture for bone growth, inherit osteogenic and functional properties (e.g., osteoinductivity and biocompatibility), optimal mechanical properties, and produce or be a vessel for bioactive factors. Although most materials fall short to possess all of these characteristics, a wide range of biomaterials are currently known and well studied for bone scaffold fabrication that presents interesting properties and biocompatibility especially when combined with materials that complement or improve the scaffold system. These materials possess multiple applications as bioactive factors delivery vessels, enhancement elements of mechanical and biological properties, or ideal structures to serve as base materials for biomimetic scaffolds. An overview of biomaterials used for the fabrication and enhancement of hierarchical scaffolds for bone tissue applications has been presented here. The several materials that have been proposed have shown great promise for BTE intending to further improve the complexity of hierarchical scaffolds. The combination of different biomaterials, fabrication procedures, and engineering structures in different dimensions are promising approaches that require further study. Fully patient-specific approaches could mean a huge step forward in regenerative medicine and CDMs are a very promising material to achieve this. The use of decellularized ECM would provide the optimal bioactive factors and architecture needed for efficient and functional biomimetic scaffolds. Noticeably, the vast majority of articles analyzed for this review merely focused on fabrication and short-term results for bone regeneration, so a translation of these potential applications to clinical testing to evaluate long-term results is needed. In brief, the complex structure of bone tissue requires a multidimensional approach that takes into account multiple structural and biological factors. Hence, to more closely mimic the natural tissue and provide better solutions for bone lesions, an increase control in complexity and effectiveness of scaffolds is necessary.

## Acknowledgements

The authors thank the funds provided by the project FROnTHERA (NORTE-01-0145-FEDER-000023), supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), the project 3BioMeD (FCT/4773/4/5/2017/S) supported by Fundação para a Ciência e a Tecnologia (FCT) and the R&D Project KOAT PTDC/BTMMAT/29760/2017 (POCI-01-0145-FEDER-029760) financed by FCT and cofinanced by FEDER and POCI. F.R.M. acknowledges FCT for her contract under the Transitional Rule DL 57/2016 (CTTI-57/18-I3BS (5)).

## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

biomaterials, bone, microparticles, nanocomposites, nanoparticles

Received: August 31, 2021
Published online:

[1] R. Agarwal, A. J. García, *Adv. Drug Delivery Rev.* **2015**, *94*, 53.
[2] Y. Kang, E. Jabbari, Y. Yang, in *Micro and Nanotechnologies in Engineering Stem Cells and Tissues* (Eds: M. Ramalingam, E. Jabbari, S. Ramakrishna, A. Khademhosseini), Wiley-IEEE Press **2013**, pp. 142–158.
[3] Y. V. Shih, S. Varghese, *Biomaterials*, **2019**, *198*, 107.
[4] T. Rozario, D. W. DeSimone, *Dev. Biol.* **2010**, *341*, 126.
[5] J. Y. Park, S. H. Park, M. G. Kim, S.-H. Park, T. H. Yoo, M. S. Kim, *Adv. Exp. Med. Biol.* **2018**, *1064*, 109.
[6] A. Wubneh, E. K. Tsekoura, C. Ayranci, H. Uludağ, *Acta Biomater.* **2018**, *80*, 1.
[7] Y. Du, J. L. Guo, J. Wang, A. G. Mikos, S. Zhang, *Biomaterials*, **2019**, *218*, 119334.
[8] L. Roseti, V. Parisi, M. Petretta, C. Cavallo, G. Desando, I. Bartolotti, B. Grigolo, *Mater. Sci. Eng., C* **2017**, *78*, 1246.
[9] J. J. Li, M. Ebied, J. Xu, H. Zreiqat, *Adv. Healthcare Mater.* **2018**, *7*, 1701061.
[10] T. Lu, Y. Li, T. Chen, *Int. J. Nanomed.* **2013**, *8*, 337.
[11] S.-J. Wang, D. Jiang, Z.-Z. Zhang, Y.-R. Chen, Z.-D. Yang, J.-Y. Zhang, Jinjun Shi, Xing Wang, Jia-Kuo Yu, *Adv. Mater.* **2019**, *31*, 1904341.
[12] H. Ma, C. Feng, J. Chang, C. Wu, *Acta Biomater.* **2018**, *79*, 37.
[13] P. Chocholata, V. Kulda, V. Babuska, *Materials*, **2019**, *12*, 568.
[14] F. Donnaloja, E. Jacchetti, M. Soncini, M. T. Raimondi, *Polymers* **2020**, *12*, 905.
[15] D. Zhang, X. Wu, J. Chen, K. Lin, *Bioact. Mater.* **2018**, *3*, 129.
[16] G. Turnbull, J. Clarke, F. Picard, P. Riches, L. Jia, F. Han, B. Li, W. Shu, *Bioact. Mater.* **2017**, *3*, 278.
[17] L. Yu, D. W. Rowe, I. P. Perera, J. Zhang, S. L. Suib, X. Xin, Mei Wei, *ACS Appl. Mater. Interfaces* **2020**, *12*, 18235.
[18] J. Wen, J. Yao, X. Chen, Z. Shao, *ACS Omega*, **2018**, *3*, 3396.
[19] J. Melke, S. Midha, S. Ghosh, K. Ito, S. Hofmann, *Acta Biomater.* **2016**, *31*, 1.
[20] F. Mottaghitalab, H. Hosseinkhani, M. A. Shokrgozar, C. Mao, M. Yang, M. Farokhi, *J. Controlled Release* **2015**, *215*, 112.
[21] B. Liu, X. Gao, Z. Sun, Q. Fang, X. Geng, H. Zhang, G. Wang, Y. Dou, P. Hu, K. Zhu, D. Wang, J. Xing, D. Liu, M. Zhang, R. Li, *J. Mater. Sci. Mater. Med.* **2018**, *30*, 4.
[22] A. C. Hernández-González, L. Téllez-Jurado, L. M. Rodríguez-Lorenzo, *Carbohydr. Polym.* **2020**, *229*, 115514.
[23] R. E. Abouzeid, R. Khiari, D. Beneventi, A. Dufresne, *Biomacromolecules*, **2018**, *19*, 4442.
[24] S. Preethi Soundarya, A. Haritha Menon, S. Viji Chandran, N. Selvamurugan, *Int. J. Biol. Macromol.* **2018**, *119*, 1228.
[25] A. Pistone, C. Celesti, E. Piperopoulos, D. Ashok, A. Cembran, A. Tricoli, D. Nisbet, *Materials* **2019**, *12*, 2321.
[26] P. Gentile, V. Chiono, I. Carmagnola, P. V. Hatton, *Int. J. Mol. Sci.* **2014**, *15*, 3640.
[27] J. Kong, B. Wei, T. Groth, Z. Chen, L. Li, D. He, R. Huang, J. Chu, M. Zhao, *J. Biomed. Mater. Res A.* **2018**, *106*, 2714.
[28] M. Xu, D. Zhai, L. Xia, H. Li, S. Chen, B. Fang, J. Chang, C. Wu, *Nanoscale* **2016**, *8*, 13790.
[29] A. A. El-Rashidy, J. A. Roether, L. Harhaus, U. Kneser, A. R. Boccaccini, *Acta Biomater.* **2017**, *62*, 1.
[30] I. D. Xynos, A. J. Edgar, L. D. K. Buttery, L. L. Hench, J. M. Polak, *J. Biomed. Mater. Res.* **2001**, *55*, 151
[31] J. S. Fernandes, R. L. Reis, R. A. Pires, *Mater. Sci. Eng., C* **2017**, *71*, 252.
[32] K. E. M. Benders, P. R. van Weeren, S. F. Badylak, D. B. F. Saris, W. J. A. Dhert, J. Malda, *Trends Biotechnol.* **2013**, *31*, 169.
[33] S. Yi, F. Ding, L. Gong, X. Gu, *Curr. Stem Cell Res. Ther.* **2017**, *12*, 233.
[34] P. M. Crapo, T. W. Gilbert, S. F. Badylak, *Biomaterials* **2011**, *32*, 3233.
[35] H. Xing, H. Lee, L. Luo, T. R. Kyriakides, *Biotechnol. Adv.* **2020**, *42*, 107421.
[36] N. Alom, H. Peto, G. R. Kirkham, K. M. Shakesheff, L. J. White, *J. Biomed. Mater. Res., Part B* **2018**, *106*, 900.
[37] M. S. Carvalho, J. C. Silva, J. M. S. Cabral, C. L. da Silva, D. Vashishth, *J. Tissue Eng. Regener. Med.* **2019**, *13*, 1544.
[38] G. M. Harris, I. Raitman, J. E. Schwarzbauer, *Methods Cell Biol.* **2018**, *143*, 97.
[39] A. Khademhosseini, R. Langer, J. Borenstein, J. P. Vacanti, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 2480.
[40] B. Yin, P. Ma, J. Chen, H. Wang, G. Wu, B. Li, Q. Li, Z. Huang, G. Qiu, Z. Wu, *Int. J. Mol. Sci.* **2016**, *17*, 575.
[41] B. Wang, W. Liu, D. Xing, R. Li, C. Lv, Y. Li, X. Yan, Y. Ke, Y. Xu, Y. Du, J. Lin, *Sci. Rep.* **2017**, *7*, 16709.
[42] D. Lin, J. Zhang, F. Bai, X. Cao, C. Fan, Y. Yuan, J. Wang, J. Zhang , C. Liu, *Am. J. Transl. Res.* **2016**, *8*, 1379.
[43] Q. Ren, M. Cai, K. Zhang, W. Ren, Z. Su, T. Yang, T. Sun, J. Wang, *Braz. J. Med. Biol. Res.* **2017**, *51*, e6520.
[44] A. Sobhani, M. Rafienia, M. Ahmadian, M.-R. Naimi-Jamal, *Tissue Eng. Regener. Med.* **2017**, *14*, 525.
[45] R. Cholas, S. Kunjalukkal Padmanabhan, F. Gervaso, G. Udayan, G. Monaco, A. Sannino, A. Licciulli, *Mater. Sci. Eng., C* **2016**, *63*, 499.
[46] P. Xia, S. Wang, Z. Qi, W. Zhang, Y. Sun, *Artif. Cells, Nanomed., Biotechnol.* **2019**, *47*, 1662.
[47] Y. Li, Z. Zhang, Z. Zhang, *Cells Tissues Organs* **2018**, *205*, 20.
[48] A. Totaro, A. Salerno, G. Imparato, C. Domingo, F. Urciuolo, P. A. Netti, *J. Tissue Eng. Regener. Med.* **2017**, *11*, 1865.
[49] N. H. Patil, P. V. Devarajan in *Colloid and Interface Science in Pharmaceutical Research and Development* (Eds: H. Ohshima, Makino KBT-C and IS in PR and D), Elsevier, Amsterdam **2014**. pp. 411–442.
[50] A. K. Goyal, T. Garg, S. Bhandari, G. Rath, in (Eds: E. Andronescu, Grumezescu AMBT-N for DD), *Micro and Nano Technologies*, **2017**, pp. 669–695.
[51] M. J. Hill, B. Qi, R. Bayaniahangar, V. Araban, Z. Bakhtiary, M. R. Doschak, B. C. Goh, M. Shokouhimehr, H. Vali, J. F. Presley, A. A. Zadpoor, M. B. Harris, P. P. S. S. Abadi, M. Mahmoudi, *Nanomedicine*, **2019**, *14*, 2987.
[52] X. Huang, S. Bai, Q. Lu, X. Liu, S. Liu, H. Zhu, *J. Biomed. Mater. Res., Part B* **2015**, *103*, 1402.
[53] C. Xie, X. Lu, L. Han, J. Xu, Z. Wang, L. Jiang, K. Wang, H. Zhang, F. Ren, Y. Tang, *ACS Appl. Mater. Interfaces*, **2016**, *8*, 1707.
[54] X. Zhou, T. Esworthy, S.-J. Lee, S. Miao, H. Cui, M. Plesiniak, H. Fenniri, T. Webster, R. D. Rao, L. G. Zhang, *Nanomedicine*, **2019**, *19*, 58.
[55] K. Qiu, B. Chen, W. Nie, X. Zhou, W. Feng, W. Wang, L. Chen, X. Mo, Y. Wei, C. He, *ACS Appl. Mater. Interfaces*, **2016**, *8*, 4137.
[56] J. M. Oliveira, R. A. Sousa, N. Kotobuki, M. Tadokoro, M. Hirose, J. F. Mano, R. L. Reis, H. Ohgushi, *Biomaterials*, **2009**, *30*, 804.
[57] T. R. Correia, D. R. Figueira, K. D. De Sá, S. P. Miguel, R. G. Fradique, A. G. Mendonça, I. J. Correia, *Int. J. Biol. Macromol.* **2016**, *93*, 1432.
[58] S. Saber-Samandari, M. Mohammadi-Aghdam, S. Saber-Samandari, *Int. J. Biol. Macromol.* **2019**, *138*, 810.
[59] L. Han, M. Wang, H. Sun, P. Li, K. Wang, F. Ren, X. Lu, *J. Biomed. Mater. Res. A.* **2017**, *105*, 3482.
[60] A. El-Fiqi, J.-H. Kim, H.-W. Kim, *Mater. Sci. Eng., C* **2020**, *110*, 110660.
[61] S. J. Lee, H.-J. Lee, S.-Y. Kim, J. M. Seok, J. H. Lee, W. D. Kim, I. K. Kwon, S.-Y. Park, S. A. Park, *Nanoscale*, **2018**, *10*, 15447.
[62] O. Guillaume, M. A. Geven, C. M. Sprecher, V. A. Stadelmann, D. W. Grijpma, T. T. Tang, L. Qin, Y. Lai, M. Alini, J. D. De Bruijn, H. Yuan, R. G. Richards, D. Eglin, *Acta Biomater.* **2017**, *54*, 386.
[63] J. Radwan-Pragłowska, Ł Janus, M. Piątkowski, D. Bogdał, D. Matysek, *Polymers*, **2020**, *12*, 159.

[64] M. Świętek, A. Brož, J. Tarasiuk, S. Wroński, W. Tokarz, A. Kozieł, M. Błażewicz, L. Bačáková, *Mater. Sci. Eng., C* **2019**, *104*, 109913.
[65] F. Norouz, R. Halabian, A. Salimi, M. Ghollasi, *Mater. Sci. Eng., C* **2019**, *103*, 109857.
[66] T.-W. Sun, W.-L. Yu, Y.-J. Zhu, F. Chen, Y.-G. Zhang, Y.-Y. Jiang, Y.-H. He, *Chem. – A Eur. J.* **2018**, *24*, 8809.
[67] V. Abbasian, R. Emadi, M. Kharaziha, *Mater. Sci. Eng., C* **2020**, *109*, 110549.
[68] M. Vedhanayagam, S. Anandasadagopan, B. U. Nair, K. J. Sreeram, *Mater. Sci. Eng., C* **2020**, *108*, 110378.
[69] X. Liu, A. L. ParkMiller 2nd, M. N. George, B. E. Waletzki, H. Xu, A. Terzic, L. Lu, *ACS Appl. Mater. Interfaces*, **2019**, *11*, 23558.
[70] Y. Murahashi, F. Yano, H. Nakamoto, Y. Maenohara, K. Iba, T. Yamashita, S. Tanaka, K. Ishihara, Y. Okamura, T. Moro, T. Saito, *Acta Biomater.* **2019**, *85*, 172.
[71] L. Han, H. Sun, P. Tang, P. Li, C. Xie, M. Wang, K. Wang, J. Weng, H. Tan, F. Ren, X. Lu, *Biomater. Sci.* **2018**, *6*, 538.
[72] H. Cao, W. Zhang, F. Meng, J. Guo, D. Wang, S. Qian, X. Jiang, X. Liu, P. K. Chu, *ACS Appl. Mater. Interfaces*, **2017**, *9*, 5149.
[73] S. Vieira, S. Vial, F. R. Maia, M. Carvalho, R. L. Reis, P. L. Granja, J. M. Oliveira, *RSC Adv.* **2015**, *5*, 77996.
[74] M. F. Griffin, D. M. Kalaskar, A. Seifalian, P. E. Butler *Open Orthop. J.* **2016**, *10*, 836.
[75] G. G. Walmsley, A. Mcardle, R. Tevlin, A. Momeni, D. Atashroo, M. S. Hu, A. H. Feroze, V. W. Wong, P. H. Lorenz, M. T. Longaker, D. C. Wan, *Nanomedicine* **2015**, *11*, 1253.
[76] J. Mariam, S. Sivakami, P. M. Dongre, *Drug Delivery* **2016**, *23*, 2668.
[77] P. Bhattacharjee, B. Kundu, D. Naskar, H.-W. Kim, T. K. Maiti, D. Bhattacharya, S. C. Kundu, *Acta Biomater.* **2017**, *63*, 1.
[78] B. N. Singh, N. N. Panda, R. Mund, K. Pramanik, *Carbohydr. Polym.* **2016**, *151*, 335.
[79] D. Ding, Q. Zhu, *Mater. Sci. Eng., C* **2018**, *92*, 1041.
[80] J. Wolfram, M. Zhu, Y. Yang, J. Shen, E. Gentile, D. Paolino, M. Fresta, G. Nie, C. Chen, H. Shen, M. Ferrari, Y. Zhao, *Curr. Drug Targets* **2015**, *16*, 1671.
[81] Y. Xia, J. Sun, L. Zhao, F. Zhang, X.-J. Liang, Y. Guo, M. D. Weir, M. A. Reynolds, N. Gu, H. H. K. Xu, *Biomaterials* **2018**, *183*, 151.
[82] S. van Rijt, P. Habibovic, *J. R. Soc. Interface* **2017**, *14*, 20170093.
[83] D. Cabuzu, A. Cirja, R. Puiu, A. M. Grumezescu, *Curr. Top. Med. Chem.* **2015**, *15*, 1605.
[84] J. Li, J. J. Li, J. Zhang, X. Wang, N. Kawazoe, G. Chen, *Nanoscale*, **2016**, *8*, 7992.
[85] S. A. Brennan, C. Ní Fhoghlú, B. M. Devitt, #F. J. O'Mahony, D. Brabazon, A. Walsh, *Bone Joint J.* **2015**, *97-B*, 582.
[86] S. Gera, S. Sampathi, S. Dodoala, *Curr. Drug Delivery* **2017**, *14*, 904.
[87] D. Pedone, M. Moglianetti, E. De Luca, G. Bardi, P. P. Pompa, *Chem. Soc. Rev.* **2017**, *46*, 4951.
[88] M. Kulkarni, A. Mazare, E. Gongadze, Š. Perutkova, V. Kralj-Iglič, I. Milošev, P. Schmuki, P. A Iglič, M. Mozetič, *Nanotechnology* **2015**, *26*, 62002.
[89] X. Li, J. Wei, K. E. Aifantis, Y. Fan, Q. Feng, F.-Z. Cui, F. Watari, *J. Biomed. Mater. Res. A.* **2016**, *104*, 1285.
[90] S. Saber-Samandari, S. Saber-Samandari, *Mater. Sci. Eng., C* **2017**, *75*, 721.
[91] Z. Peng, T. Zhao, Y. Zhou, S. Li, J. Li, R. M. Leblanc, *Adv. Healthcare Mater.* **2020**, *9*, 1901495.
[92] B. Pei, W. Wang, N. Dunne, X. Li, *Nanomaterials* **2019**, *9*, 1501.
[93] R. Eivazzadeh-Keihan, A. Maleki, M. De La Guardia, M. S. Bani, K. K. Chenab, P. Pashazadeh-Panahi, B. Baradaran, A. Mokhtarzadeh, M. R. Hamblin, *J. Adv. Res.* **2019**, *18*, 185.
[94] S. Gholizadeh, F. Moztarzadeh, N. Haghighipour, L. Ghazizadeh, F. Baghbani, M. A. Shokrgozar, Z. Allahyari, *Int. J. Biol. Macromol.* **2017**, *97*, 365.
[95] N. Shadjou, M. Hasanzadeh, *Mater. Sci. Eng., C*, **2015**, *55*, 401.
[96] J. Wang, L. Wang, Y. Fan, *Int. J. Mol. Sci.* **2016**, *17*, 798.
[97] T. J. Levingstone, S. Herbaj, N. J. Dunne, *Nanomaterials* **2019**, *9*, 1570.
[98] Y. Chen, N. Kawazoe, G. Chen, *Acta Biomater.* **2018**, *67*, 341.
[99] B. N. Singh, K. Pramanik, *J. Biomater. Sci. Polym. Ed.* **2018**, *29*, 2011.
[100] B. Gorain, M. Tekade, P. Kesharwani, A. K. Iyer, K. Kalia, R. K. Tekade, *Drug Discov. Today* **2017**, *22*, 652.
[101] J. M. Oliveira, N. Kotobuki, A. P. Marques, R. P. Pirraco, J. Benesch, M. Hirose, S. A. Costa, J. F. Mano, H. Ohgushi, R. L. Reis, *Adv. Funct. Mater.* **2008**, *18*, 1840.
[102] J. Bramhill, S. Ross, G. Ross, *Int. J. Environ. Res. Public Health*, **2017**, *14*, 66.
[103] A. Bharadwaz, A. C. Jayasuriya, *Mater. Sci. Eng., C* **2020**, *110*, 110698.
[104] S. Pina, J. M. Oliveira, R. L. Reis, *Adv. Mater.* **2015**, *27*, 1143.
[105] C. Covarrubias, A. Agüero, M. Maureira, E. Morelli, G. Escobar, F. Cuadra, C. Peñafiel, A. Von Marttens, *Mater. Sci. Eng., C*, **2019**, *96*, 642.
[106] K. Muazim, Z. Hussain, *Mater. Sci. Eng., C* **2017**, *76*, 1274.
[107] S. Thurakkal, X. Zhang, *Adv. Sci.* **2019**, *7*, 1902359.
[108] Y. Qing, R. Li, S. Li, Y. Li, X. Wang, Y. Qin, *Int. J. Nanomed.* **2020**, *15*, 2045.
[109] J. Jeong, J. H. Kim, J. H. Shim, N. S. Hwang, C. Y. Heo, *Biomater. Res.* **2019**, *23*, 4.
[110] G. Kaur, V. Kumar, F. Baino, J. C. Mauro, G. Pickrell, I. Evans, O. Bretcanu, *Mater. Sci. Eng., C* **2019**, *104*, 109895.
[111] S. Ranganathan, K. Balagangadharan, N. Selvamurugan, *Int. J. Biol. Macromol.* **2019**, *133*, 354.
[112] S. Hassanajili, A. Karami-Pour, A. Oryan, T. Talaei-Khozani, *Mater. Sci. Eng., C* **2019**, *104*, 109960.
[113] S. Vieira, S. Vial, R. L. Reis, J. M. Oliveira, *Biotechnol. Prog.* **2017**, *33*, 590.
[114] G. Turnbull, J. Clarke, F. Picard, P. Riches, L. Jia, F. Han, B. Li, W. Shu, *Bioact. Mater.* **2018**, *3*, 278.
[115] Q. Zhu, X. Li, Z. Fan, Y. Xu, H. Niu, C. Li, Y. Dang, Z. Huang, Y. Wang, J. Guan, *Mater Sci. Eng., C* **2018**, *85*, 79.
[116] S. Injamuri, M. N. Rahaman, Y. Shen, Y.-W. Huang, *J. Biomed. Mater. Res. A.* **2020**, *108*, 1231.
[117] J. R. Choi, K. W. Yong, J. Y. Choi, A. Nilghaz, Y. Lin, J. Xu, X. Lu, *Theranostics* **2018**, *8*, 1005.
[118] P.-C. Chang, B.-Y. Liu, C.-M. Liu, H.-H. Chou, M.-H. Ho, H.-C. Liu, D.-M. Wang, L.-T. Hou, *J. Biomed. Mater. Res. A* **2007**, *81*, 771.

**Rafael Lemos** studied genetics and biotechnology at University of Trás-os-Montes e Alto Douro where he worked on genetic characterization of pinus pinaster followed by a brief period as a research trainee in the 3B's Research Group—University of Minho. He has a master's degree in biophysics and bionanosystems (University of Minho) during which he worked on novel carbon nanotubes-reinforced cell-derived matrix-silk fibroin hierarchical scaffolds for bone tissue engineering applications.

**F. Raquel Maia** obtained her Ph.D. degree in biomedical engineering, which was approved with distinction from the Faculty of Engineering of the University of Porto. During this time she studied the use of nanostructured 3D natural polymeric matrices capable to direct mesenchymal stem cells behavior. Then, she became a postdoc fellow at 3B's Research Group where she used her knowledge in tissue engineering to develop new approaches for cancer and osteoarthritis research. Currently, F. R. Maia is a junior investigator at University of Minho focused on the development of bioengineered cell-derived matrices envisioning their use on modeling bone tissue's microenvironment.

**Rui L. Reis** Ph.D., D.Sc., Hon. Causa MD, FBSE, FTERM, member NAE (USA), is a full professor of tissue engineering, regenerative medicine and stem cells, and the director of the Ph.D. program on tissue engineering, regenerative medicine and stem cells of University of Minho. He is also the director of the 3B's Research Group and of the PT Associate Laboratory ICVS/3B's, of University of Minho. He is the CEO of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, and the editor-in-chief of the Journal of Tissue Engineering and Regenerative Medicine.

**J. Miguel Oliveira** is a biochemist and principal investigator with habilitation at 3Bs Research Group. He is the Vice-President of the Institute 3Bs at University of Minho. He is lecturer in Doctoral Program in Tissue Engineering, Regenerative Medicine and Stem Cells at University of Minho. He has been awarded 21 prizes/honors, being the most prestigious one, The Jean Leary Award 2015 (Young Scientists and Group Leaders under 40 years old) attributed by the European Society for Biomaterials for its outstanding contributions within the field of biomaterials. J. M. Oliveira is one of the Founder Editors-in-chief of In vitro Models (Springer).