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Nano-Doped Matrices for Tissue Regeneration

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1. Introduction

Tissue engineering can be defined as an interdisciplinary field applying the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function (Langer & Vacanti, 1993). To accomplish this aim, a balanced combination of cell culture growth with supporting biomaterials is required, as well as the introduction of bioactive agents able to enhance and direct cell and tissue aggregation. Regenerative medicine has been sometimes looked as an extension of tissue engineering, but it can be considered nowadays one of the major interdisciplinary scientific challenges, aiming at regenerating the soft and hard tissues, organs, and nerves damaged or responsible for main human disabilities. The definition given by Kaiser marks the difference of such field from others, and its specific link with pathologies care: "*a new branch of medicine will develop that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems*" (Kaiser, 1992). The central focus of regenerative medicine is represented by human cells. These may be somatic, adult or embryo-derived stem cells and, recently, induced-pluripotent stem cells.
In both tissue engineering and regenerative medicine, the role of scaffolds is predominant, constituting the framework for cell attachment, proliferation, and differentiation. Biodegradable natural and synthetic polymers, as well as some non-biodegradable polymers have been extensively studied and used for the 2D and 3D reconstruction, as well as for the healing of different tissue typologies (Jagur-Grodzinski, 2006). Several requirements have been identified as crucial for the production of tissue engineering scaffolds: (1) the scaffold should possess interconnecting pores of appropriate scale to favour tissue integration and vascularisation; (2) it should be made from material with controlled biodegradability or bioresorbability, allowing the new forming tissue to replace the scaffold; (3) it should have appropriate surface chemistry to favour cellularattachment, proliferation and differentiation; (4) it should own adequate mechanical properties to match the intended site of implantation and handling; (5) it should not induce any adverse response; (6) it should be easy to fabricate into a variety of sizes and shapes (Hutmacher, 2001). Furthermore, it is known that the principal objective of a scaffold is to recapitulate extracellular matrix (ECM) function in a temporally coordinated and spatially organized structure, and a key issue is to encode required biological signals within the scaffold, in order to control the main cellular processes (Causa *et al*., 2007).
In order to mimick the nanometric organization of biological structures, many attempts have been made in the latest years in order to obtain synthetic or natural scaffolds having

nanometric-sized cues, able to better direct cell behaviour. Nanotechnologic processes paved the way for such challenge, allowing for the preparation of matrices with pores, grooves, fibers and other structures in a submicrometric range of sizes. This allowed a broad range of new insights, like the investigation of the effect of submicron topography on cell adhesion, migration and differentiation, or the evaluation of the differences, in terms of protein adsorption and morphology, on materials showing a nanometric roughness in comparison with more flat or micrometric-roughness-characterized substrates. Furthermore, the introduction of nanocomposites made by bulk materials (*e.g.*, polymers) with nanoparticles embedded in their structure, allowed the fabrication of new structured nanoscale materials having promising properties for tissue engineering applications. In general, the ability to control the assembly of nanoparticles into discrete organized clusters inside a polymeric matrix is of broad interest in the field of nanotechnology, as magnetic, electronic and optical behaviours of complex, three-dimensional nanocomposites are highly dependent on the size of the nanoparticles, as well as on the distance between them (Sanyal *et al*., 2004). Such nanoparticle-mediated properties become biologically interesting when integrated into a scaffold for tissue engineering or regenerative purposes; the incorporation of nano-sized objects into degradable or not-degradable polymeric networks, in fact, may provide a more favourable synthetic microenvironment to more closely mimic natural tissue physiology, with the addition of certain physical stimuli.

In the present chapter, some techniques and preparation methods to obtain nano-doped matrices are described, together with the chemical and physical properties of the fabricated nanoscale material. The encouraging results obtained by many research groups using polymeric scaffolds doped with various nanoparticle typologies are then reported, highlighting the specific advantages that the inclusion of such nanoparticles brings to this technology. The main aspects regarding protein adsorption, cell adhesion, proliferation and differentiation on the described scaffolds are discussed, also trying to envision future applications and challenges related to these materials. The conclusion aims at strengthening what emerges from recent studies, namely that nano-doped scaffolds can be considered a new and promising instrument for cell and tissue growth and regeneration, with the possibility to provide tuned and controlled physical stimuli to the biological culture, in order to enhance or direct its behaviour.

2. The importance of "nano" in scaffold design

Nanomaterials are materials with basic structural units, grains, particles, fibers or other constituent components smaller than 100 nm in at least one dimension (Siegel & Fougere, 1995), and they include nanoparticles, nanoclusters, nanocrystals, nanotubes, nanofibers, nanowires, nanorods, nanofilms, *etc.* The intrigue of nanotechnology relies on the ability to control material properties by assembling such materials at the nanoscale, allowing for the construction of new devices with peculiar properties. In fact, after decreasing material size into the nanoscale, there is a dramatic increase in surface area and surface roughness to volume ratios, thus leading towards superior physiochemical (*i.e.*, mechanical, electrical, optical, catalytic, magnetic, *etc.*) properties.

Several investigators recently came to the conclusion that in order to achieve a breakthrough in the fields of tissue engineering and regenerative medicine, it is necessary to mimic the natural biological processes (Tu & Tirrell, 2004; Stupp, 2005). From this perspective, the importance of nanomaterials becomes clear, since natural tissues or organs are nanometer in

dimensions and cells directly interact with nanostructured ECM matrix. Bone, for example, is a nanocomposite consisting of a protein based soft hydrogel template (constituted by collagen or other non-collagenous proteins (laminin, fibronectin, vitronectin, *etc.*) and water) and hard inorganic components like hydroxyapatite (HA). The bone matrix is composed for the 70% of nanocrystalline HA, which is typically 20-80 nm long and 2-5 nm thick (Kaplan *et al*., 1994). Other protein components in the bone ECM have also nanometric size, contributing to create a nanoscale environment surrounding and affecting mesenchymal stem cell, osteoblast, osteoclast, and fibroblast adhesion, proliferation and differentiation. Cartilage is a flexible connective tissue composed of a small cellular component (the chondrocytes) and a dense nanostructured ECM rich in collagen fibers, proteoglycans and elastin fibers. Cartilage is able to lubricate joints and withstand static and dynamic loads remarkably well; such properties can be well understood treating cartilage ECM as a composite medium, with a proteoglycans phase exerting swelling pressure and collagen phase resisting it (Basser & Horkay, 2005).

The use of nanomaterials for bone and cartilage regeneration allowed the achievement of exciting results, well reviewed in (Zhang & Webster, 2009). This success is due to the dimensional similarity of materials to bone/cartilage tissue, but also to their special surface properties (topography, surface wettability, and surface energy) due to their greater surface area and roughness compared to conventional or micron structured materials. An important mechanism underlying the increased cellular response on nanostructured materials relies in protein adsorption. As known, cell behaviour is strongly influenced by serum protein adsorption on the biomaterials surface; a nanostructuration allows an increased adsorption of specific proteins (fibronectin, vitronectin, laminin, *etc.*) before cells adhere on implants, regulating cell behaviour and dictating tissue regeneration. Furthermore, the surface topography determines not only the amount of protein adsorbed, but even its functional interconnectivity (Fig. 1).

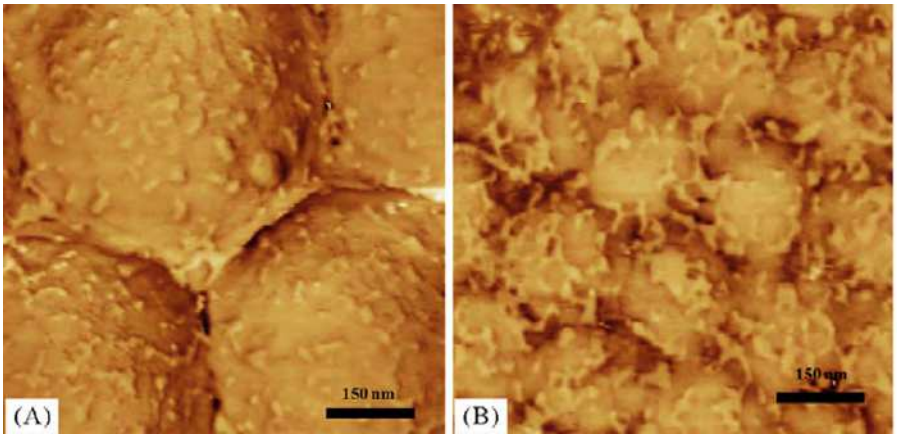

Fig. 1. Atomic force microscope of PLGA nanospheres with different dimensions coated with fibronectin (5 μg/ml). (A) Phase image of fibronectin adsorbed on PLGA with 500 nm surface features, revealing no interconnectivity between proteins. (B) Phase image of fibronectin adsorbed on PLGA with 200 nm surface features, revealing significant interconnectivity between proteins. Images from (Miller *et al*., 2007). Reproduced with permission from John Wiley and Sons.

Collagen in bone and cartilage is a triple helix self-assembled into nanofibers 300 nm in length and 1.5 nm in diameter; many recent efforts have been therefore dedicated to exploring the influence of novel biomimetic nanofibrous or nanotubular scaffolds on regenerative medicine, by following a bottom-up self-assembly process. Carbon nanotubes/nanofibers (CNTs/CNFs), for example, due to their superior mechanical and electrical properties, are ideal scaffold candidates for bone tissue engineering applications (Tran *et al*., 2009).

Vascular tissue is a layered structure characterized by several nanostructured feature, due to the presence of collagen and elastin in the vascular ECM. Even for this kind of tissue, nanomaterials have a strong influence on regeneration performances, like demonstrated by (Choudhary *et al*., 2007), reporting that vascular cell adhesion and proliferation were greatly improved on nanostructured Ti compared to conventional Ti.

Nanomaterials have also demonstrated to be useful for damaged nerves healing. Central nervous system (CNS) and peripheral nervous system (PNS) show different repair procedures after injury: for the PNS, the damaged axons usually regenerate and recover by means of proliferating Schwann cells; for the CNS, it is much more difficult to re-extend and re-innervate axons, due to the absence of Schwann cells. Moreover, due to the influence of astrocytes, meningeal cells and oligodendrocytes, a thick glial scar tissue forms around neural biomaterials, preventing proximal axon growth and inhibiting neuron regeneration. To be effective in PNS and CNS regeneration, biomaterials should therefore have excellent cytocompatibility, mechanical and electrical properties. Nanotechnology allowed in the latest years the development of novel and improved neural tissue engineering materials, with the design of nanofiber/nanotube scaffolds (Fig. 2) with exceptional cytocompatibility and conductivity properties to boost neuron activities (Mattson *et al*., 2000; Gheith *et al*., 2005), as well as with the introduction of piezoelectric nanoparticles-mediated neuron stimulation, with the aim of enhancing neurite outgrowth in treated cells (Ciofani *et al*., 2010).

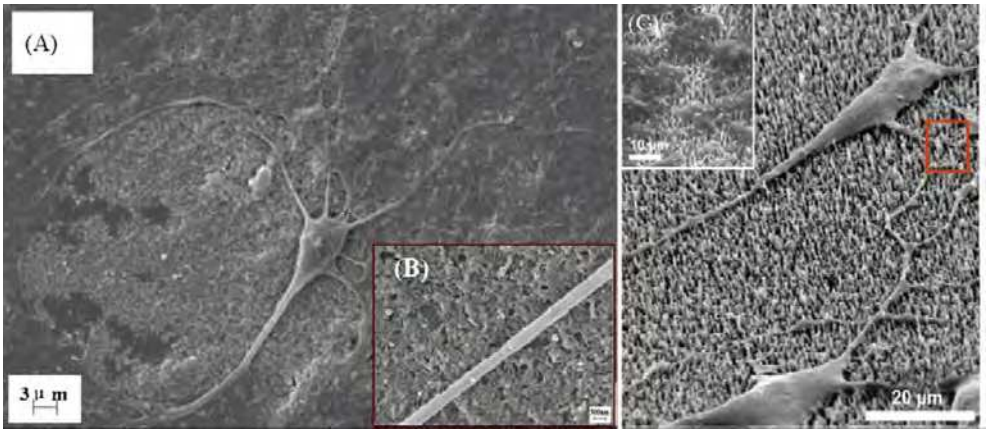

Fig. 2. a Neonatal hippocampal neurons adhering on purified multi-walled carbon nanotubes-coated glass substrates, showing extended neuritis after 8 days of culture; (B) single neurite in close contact to carbon nanotubes; (C) PC12 neural cells grown on vertically aligned carbon nanotubes coated with polypyrrole. Images from (Zhang & Webster, 2009). Reproduced with permission from Elsevier.

The regeneration of other tissues and organs, like bladder, muscle, skin, kidney, liver, pancreas and immune system has been addressed using nanotechnology approaches (Nukavarapu, *et al*., 2008; Khademhosseini *et al*., 2008). Nanomaterials more efficiently improve such tissues regeneration, for the same reasons mentioned above (biologically inspired roughness, increased surface energy, selective protein adsorption, *etc.*).

3. Preparation methods and properties of doped matrices

Composite materials made of nanoparticles in a polymeric matrix have been under investigation for at least one decade, not only for biological applications (only recently proposed), but also to develop smart devices, like nonlinear optical materials. A study of Pavel and MacKay (Pavel & Mackay, 2000) described the production of randomly dispersed (and non-aggregated) cadmium sulfide nanoparticles in a transparent polymer matrix of poly(methyl metacrylate) (PMMA). The process was based on a reverse micellar system, followed by polymerization to produce a solid inorganic/organic composite, and it was the first report in the literature of such technique to obtain a "one-system" synthesis of nanoparticles dispersed in a polymer matrix.

In general, the field of polymer matrix-based nanocomposites started to emerge with the recognition that exfoliated clays could yield significant mechanical properties advantages as a modification of polymeric systems. Clays are naturally occurring minerals, mainly aluminosilicates, having a sheet-like (layered) structure, and consisting of silica SiO_4 tetrahedra bonded to alumina AlO_6 octahedra in a variety of ways. Nanocomposites can be obtained by following different methods, such as in situ polymerization, solution, and latex methods. Melt processing has been also recognized an appreciable method, as it is considered more economical, more flexible for formulation, and it involves compounding and fabrication facilities commonly used in commercial practice (Paul & Robeson, 2008). During the fabrication process, a key aspect in the polymer-clay (or organoclay) interaction is the affinity that the polymer segments have for the silicate surface. Specific surfactants can be used, in order to induce a greater exfoliation; in all cases, the best exfoliation is achieved when the structure of the surfactant and the process parameters are optimized (Fig. 3(A)). As already mentioned, the most evident effect of clay-based reinforcement is an increase of the mechanical properties of the material, that has been demonstrated to be superior if compared with microcomposites or bulk materials (Fig. 3(B)).

Electrophoretic deposition (EDP) is a cost-effecting and efficient processing method to produce ceramic nanocomposites and laminates. It can be seen as two combined processes: first the migration of charged particles dispersed in a liquid medium (electrophoresis) under an applied electric field and secondly the coagulation process of the particles at the electrode. Good dispersion and stability of the suspension are essential factors to obtain a homogeneous deposition. Cho and co-workers described the co-deposition of carbon nanotubes (CNTs) and TiO_2 nanoparticles on a stainless steel substrate by EDP (Cho *et al*., 2008). During this process, both CNTs and titania particles move towards the deposition electrode, once an electrical field is applied, creating a region close to the electrode surface, specifically called "infiltration trajectory". Here, the charge of the deposited CNT films influences the motion of the charged particles; under the effect of the repulsive forces due to the surrounding CNTs, the particles will follow the path with the fewest possible obstacles until reaching the next interstice between adjacent CNTs. The result is a homogeneous distribution of CNTs and TiO_2 throughout the whole coating thickness, with a high amount

of porosity. The authors did not perform quantitative evaluation of physical properties, but they envisioned the interesting mechanical, electrical conductivity, and photocatalytic properties of these coatings, useful for biomedical applications.

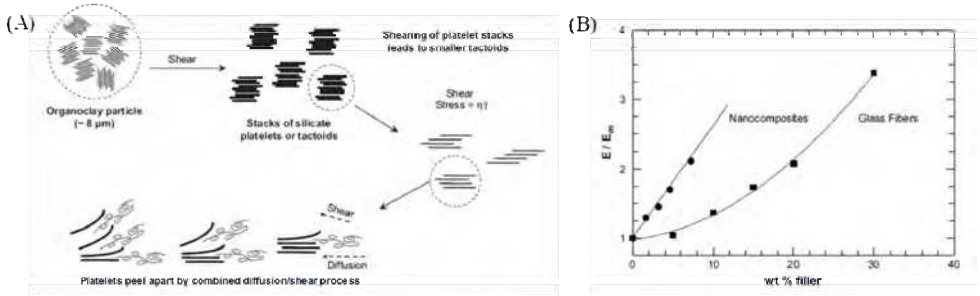

Fig. 3. a Mechanism of organoclay dispersion and exfoliation during melt processing; (B) comparison of modulus reinforcement (relative to matrix polymer) between nanocomposites based on nanoclays and glass fibers (aspect ratio ~20) for a nylon 6 matrix. Images from (Paul & Robeson, 2008). Reproduced with permission from Elsevier.

In order to replicate natural structures on the nano-scale level, electrospinning is a common and inexpensive method that allows the easy fabrication of random or ordered nanofibrous constructs. A variety of materials can be processed by electrospinning, and a certain level of control can be achieved over the desired nano-structures. In the development of bone grafts, nanofibrous scaffolds have been used as fillers, and to bridge critical size defects; the fabrication of nanofiber-calcium phosphate composites has been a step forward. Since minerals in the native bone are predominantly found on the surface, some researchers have deposited hydroxyapatite nanoparticles on the surface of electrospun fibers. The most commonly used surface mineralization techniques include soaking the scaffold in simulated body fluid (SBF) (Wei & Ma, 2006) and the alternating dipping method (Ngiam *et al*., 2009). Of these two techniques, the alternating dipping method has the advantage of significantly faster mineral deposition, requiring hours to complete rather than days for the SBF soaking technique.

The mineralization of 3D block nanofibrous scaffolds is different. In fact, 2D membranes have most part of their surface exposed to the solution, while 3D block scaffolds require the solution to reach the inner core, requiring a modification of the alternating dipping technique. Teo and co-workers described the fabrication of 3D electrospun poly(L-lactic acid) scaffolds and their mineralization by means of two approaches: the static mineralization (soaking the scaffold for 1h in a mineral solution) and the flow mineralization (placing the scaffold in a perfusion chamber, and using a circulating peristaltic pump to periodically provide the scaffold with the mineral solution) (Teo *et al*., 2011). The analysis of mechanical properties revealed that the presence of minerals significantly increased both compressive strength and compressive modulus of the scaffolds. The compressive strength of the static mineralized scaffolds was 2.4 times greater than that of the unmineralized scaffolds, while the modulus was 1.9 times greater. Such analysis also revealed that the mineralization technique has a significant influence on the mechanical properties of the scaffolds, being the compressive strength and modulus of the flow mineralized scaffolds 6.1 times and 2.8 times greater, respectively, than those of the

static mineralized scaffolds. Interestingly, these properties are not related to the overall mineral content (found to be 43.3% in flow mineralized scaffolds, and 62.3% in static mineralized scaffolds), but to the distribution of minerals inside the 3D structure of the scaffold.

The use of nanoparticles (mainly nano-hydroxyapatite), nanofibers and nanotubes in polymer or bioceramic matrices in order to produce nanometric features on the surface of 3D scaffolds has been recently well reviewed (Meng *et al*., 2010). Table 1 shows advantages and disadvantages of the main used techniques to produce nanocomposite scaffolds.

Nanostructured surfaces	**Method of fabrication**	**Advantages**	**Disadvantages**
Nanoparticles	Solvent-casting/salt-leaching technique	Controlled porosity, controlled pore size and simple operation	Limited interpore connectivity, using organic solvents
	Modified rapid prototyping technique	Ability to produce complex products quickly	Difficulty to design and fabricate scaffolds with fine microstructures, low porosity
	Freeze-drying and freezing -lyophilization	Highly porous structures, high pore interconnectivity	Limited to small pore sizes
	Self-dispersing technique	Good dispersion, immobilization of nanoparticles on the surface of 3D scaffolds by chemical bonding	Relatively long processing time, use of organic solvents
	LbL method	Multilayers incorporating proteins, drugs, and growth factors in mild conditions, strong adhesion between each layer, easy to control many processing variables during the preparation	Dissolving problems of nanoparticles in aqueous media, requiring organic solvents
	Paste extruding deposition Process	Complete pore interconnectivity, macroshape control, 3D interconnected pore structure	Limited to small pore sizes, use of organic solvents
	Micro stereolithography MSTL	Controlling the shape of composite structures with higher resolution, well developed 3D interconnected pore structure	Shrinkage problems

Nanostructured surfaces	Method of fabrication	Advantages	Disadvantages
	Layer manufacturing	Well-interconnected pores, improved surface roughness	Use of organic solvents, problems with residual solvent
	Nano-emulsion and selective laser sintering techniques	Nanosized particles are well encapsulated in microspheres, complete pore interconnectivity, macroshape control	High processing temperatures, limited to small pore sizes
	TIPS and subsequent solvent Sublimation	High porosity with pore anisotropy and high pore interconnectivity	Use of organic solvents and long time to sublime solvent
Nanofibers	Particle leaching and Electrospinning	Homogeneous nanofiber morphology can be generated spontaneously in a 3-D macroporous and nanofibrous structure	Relatively long processing time, problems with residual solvent
	Fiber bonding and Electrospinning	Combination of nano and microfibers in the same 3D scaffold architecture, mimicking the physical structure of ECM	Limit range of polymers, use of organic solvents
	Electrospinning and LbL Methods	Controlled fiber layer thickness, fiber diameter, and fiber orientation, ability to create complex hierarchical architectures	Use of high-voltage apparatus
	DPMD and electrospinning	Ability to fabricate highly functionalized 3D scaffolds with an open porous network, a controllable shape and a biocompatible nanofibrous inner architecture	Relatively high processing temperature for the workable range
	Electrospinning with 3D Collecting	Controlled patterned architectures and 3D configurations	Difficulties to control many parameters
	Phase separation and particle leaching techniques	Controlled macropore shape and size by particles, interpore opening size by assembly conditions, pore wall morphologies by phase separation parameters	Limited to a few polymers, longer processing time, unable to produce long and continuous fibers with control over fiber orientation

Nanostructured surfaces	Method of fabrication	Advantages	Disadvantages
	Self assembly	Close resemblance to biological processes	Complex process, limited to a few polymers, inability to control
CNT assemblies	EPD	Simple process, homogeneous nano and microstructures possible, controlled thickness of CNT layers in different substrates	Needs to use organic solvents in some cases
	Chemical modification	Controlled surface chemistry, good dispersion, direct covalent bonding with the matrix	Use of organic solvents
	Solvent casting with the inclusion of CNTs	Achieves mechanical integrity, electrical conductivity	Limited by the complexity in the design, construction of the mold

Table 1. Methods of fabrication of 3D nanocomposite scaffolds and corresponding advantages and disadvantages of each strategy. Table from (Meng *et al*., 2010). Reproduced with permission from John Wiley and Sons.

The hybrid technique developed by Park and co-workers, based on a combination of direct polymer melt deposition (DPMD) and electrospinning (Park *et al*., 2008) is particularly interesting, even if not including nanoparticles but nanofibers in a polymer matrix. The aim of the DPMD process was to determine the overall architecture of the 3D scaffold, by producing polycaprolactone (PCL) microfibers able to provide mechanical support and overall shape of the device; to this aim, a stainless steel syringe with a micronozzle, a syringe heating device, a compressed air dispenser and a three-dimensionally moving micropositioning system were used. The electrospinning process allowed to obtain PCL and PCL/collagen nanometric fibers applying voltage to a 22-gauge needle on a syringe pump at an infusion speed of 10 μm/min and using a grounded collector. By combining DPMS and electrospinning a hybrid process was proposed, consisting of two-step sequential process (Fig. 4(a)). As a woodpile-like structure was fabricated in a layer-by-layer approach, the nanofiber matrices were inserted between each layer of the microfibrous structure. Subsequent microfiber layers combined with nanofiber matrices were repeatedly laminated onto the previously combined layers, in order to fabricate a 3D complex structure. (Fig. 4(B) and 4(C)).

The beauty of this and other similar approaches is the ability to efficiently simulate the 3D fibrous extracellular environment, allowing the envisioning of challenging applications in tissue engineering and regeneration.

Another hybrid approach was proposed by Erisken and co-workers, that described a methodology integrating a twin screw extrusion process with electrospinning, and allowing the dispersion of nanoparticles into polymeric binders and the generation of nanoparticle-incorporated fibers and nanofibers. In particular, they dispersed β-tricalcium phosphate (β-

TCP) nanoparticles into PCL matrices, in order to generate biodegradable non-woven meshes for tissue engineering applications (Erisken *et al.*, 2008-a). The fiber size was in the range 200 – 2000 nm, and mechanical characterization of the scaffolds revealed that the ultimate tensile strength at break of the meshes increased from 0.47 to 0.79 upon the incorporation of the β-TCP nanoparticles.

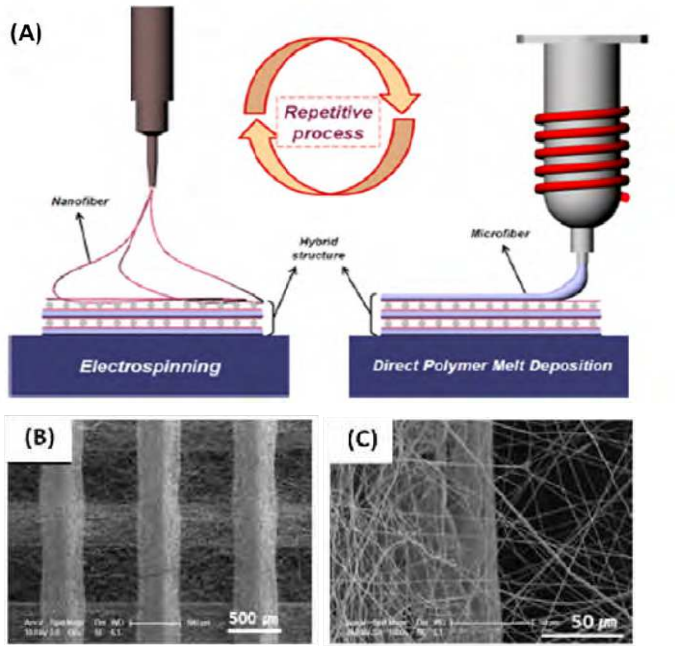

Fig. 4. (A) The DPMD/electrospinning hybrid process. A 3D structure can be built by alternating such processes; (B) and (C) photographs of hybrid layers composed by microfibers and electrospun nanofibers. Images from (Park *et al.*, 2008). Reproduced with permission from Elsevier.

More recently, Jack and co-workers reported the fabrication and characterization of nano-sized hydroxyapatite (HA)/poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) polymer composite scaffolds, developed by means of a modified thermally induced phase-separation technique (Jack *et al.*, 2009). The presence of the HA nanoparticles significantly affected the thermodynamic state of the polymer-solvent solutions, resulting in a completely different porous architecture upon solidification. EDS characterization confirmed that the clusters observed in the SEM images were indeed HA particles (Fig. 5(A), (B), and (C)).
In order to investigate the mineralization ability, the scaffolds were immersed in SBF for an overall period of 2 weeks, evaluating the mechanical performances before and after this treatment. The results showed that the immersion of the pure PHBV scaffolds in SBF for up to 2 weeks does not significantly change their mechanical performances, confirming that the polymer matrix does not significantly degrade in such an environment over the observed period. In addition, the incorporation of the HA nanoparticles leads to a marked increase in both the stiffness and strength of the scaffolds (Fig. 5(D) and (E)).

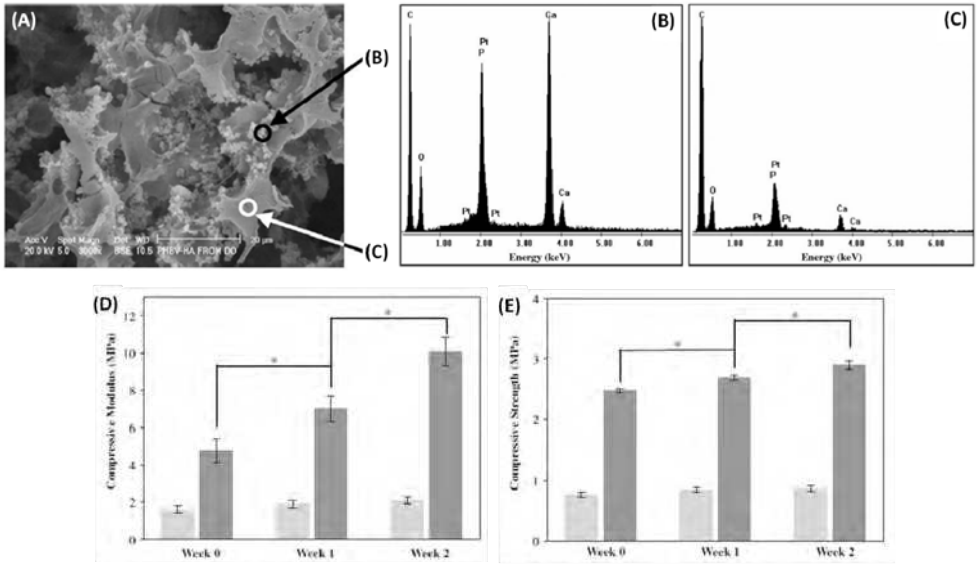

Fig. 5. (A) SEM image showing the PHBV scaffold doped with HA nanoparticles. The circles indicate the approximate regions in the scaffold that the EDS spectra (B) and (C) were collected from; compressive moduli (D) and compressive strength (E) of the PHBV (light grey) and HA/PHBV (dark grey) scaffolds following 0, 1 and 2 weeks of immersion in SBF. *=p<0.05. Images from (Jack *et al*., 2009). Reproduced with permission from Elsevier.

As described, biocompatibility and structural stability are two important aspects for engineering functional tissues. An interesting grafted collagen scaffold with a collagen triple-helix shape was developed and reinforced with Al_2O_3-ZrO_2 nanoparticles, in order to strengthen the mechanical properties of the matrix (Cao *et al*., 2006). Such modification also enhanced the thermal stability of the collagen matrix, bringing the authors to envision biomedical and bionic applications of this hybrid material. Regarding the influence of nanoparticles inclusion on the degradability of a matrix, Liu and co-workers showed that titania nanoparticles alter PLGA degradation, when dispersed in the polymeric structure (Liu *et al*., 2006). To produce the scaffolds, PLGA pellets were dissolved in chloroform and nanophase titania powder was added to give a 30/70 ceramic/polymer weight ratio. The composite mixture was then sonicated using different output power settings (and thus obtaining different samples). After sonication, the suspension was cast into a Teflon petri dish, evaporate in air at room temperature for 24 h and dried in air vacuum chamber for 48 h. After incubation in PBS for several days, the weight loss for the doped samples was significantly lower than that of pure PLGA samples. Moreover, results showed that the nanophase titania dispersed in PLGA can improve the structural stability and buffer the harmful pH variations.

4. Current applications in tissue engineering and regenerative medicine

The development of innovative materials useful as scaffolds for regenerative medicine and tissue engineering purposes tries to address, as we described, the mimicking of natural ECM

in terms of structure, chemical composition, and mechanical properties. In the latest decade, an intense research on polymeric matrices doped with several kinds of nanoparticles has been carried on, trying to focus on the possible *in vitro* and *in vivo* biomedical applications of these materials. For example, an interesting electrically conductive biodegradable composite material made of polypyrrole (PP) nanoparticles and poly(D,L-lactide) (PDLLA) was synthesized in 2004 and tested *in vitro* with human skin fibroblasts (Shi *et al*., 2004). It is well known that electrical stimulation is capable of modifying cellular activities, like cell migration, cell adhesion, DNA synthesis, and protein secretion. An electrically conductive scaffold is therefore interesting for the regulation of these activities, but it also must satisfy other basic features, as biocompatibility and degradability. Such aim was achieved in this study, including electrically conductive PP nanoparticles in a biocompatible and degradable PDLLA matrix, easy to process and to test with cell cultures. Human skin fibroblasts were cultured on the doped scaffolds, applying electrical currents in the range 0-800 μA. Cell growth was affected by such stimulation, showing a maximum peak of growth in correspondence to a 10 μA current stimulation.

The development of doped matrices can be useful not only for tissue regeneration, but also for the enhancement of materials antimicrobial acitivity. It is the case of the cellulose acetate nanofibers containing silver nanoparticles produced by Son and co-workers. They demonstrated for the first time that polymer nanofibers containing Ag nanoparticles on their surface could be produced by UV irradiation of the elctrospun fibers with small amounts of silver nitrate ($AgNO_3$) (Son *et al*., 2006). The number and size of Ag nanoparticle on the matrix surface was found to continuously increase with the irradiation time, and that the particles with an average size of 21 nm exhibited a strong antimicrobial activity.

Specific applications for bone tissue engineering were described by some works in 2006 and 2007, reporting the results obtained by fabricating doped matrices and testing their biological activity by immersing them in standard culture media and simulated body fluid (SBF), or evaluating their *in vitro* response with osteoblasts-like cells or with human mesenchymal stem cells (hMSCs) (Li *et al*., 2006; Gerhardt *et al*., 2007; Sui *et al*., 2007; Torres *et al*., 2007; Zhang *et al*., 2007). The first example of Li *et al*. is constituted by electrospun silk fibroin scaffolds containing bone morphogenetic protein 2 (BMP-2) and nanoparticles of hydroxyapatite. TEM images confirmed that nHAP particles were successfully embedded in the silk fiber scaffold, being well oriented along the fiber axis in some regions, but more aggregated in other regions. Human bone marrow-derived MSCs were cultured for up to 31 days on nHAP- and BMP-2-containing silk scaffolds, on only BMP-2-containing silk scaffolds, and on bare silk scaffolds as control, using an osteogenic medium. The scaffolds containing BMP-2 supported higher calcium deposition and enhanced transcript levels of bone-specific markers than on the controls; furthermore, the coexistence of BMP-2 and nHPA in the scaffold resulted in the highest calcium deposition and up-regulation of the transcript levels of the bone-related markers. Gerhardt and co-workers developed PDLLA scaffolds filled with 0, 5 and 30 wt% TiO_2 nanoparticles by means of solvent casting. They showed that the titania nanoparticles were not directly exposed on the composite surface, but rather embedded in the PDLLA matrix, without forming agglomerates even at the highest concentration. The bioactivity of such scaffolds was evaluated by immersing them in supersaturated simulated body fluid for up to 3 weeks and evaluating the formation of

hydroxyapatite on the material surface. HA nanocrystals with an average diameter of 40 nm were formed on the 30 wt% TiO_2 composite films after 2 weeks, while only some traces of HA crystals appeared on pure PDLLA and low titania content films after 3 weeks. The effect of TiO_2 nanoparticles on the metabolic activity of MG-63 osteoblast-like cells was also evaluated, finding that particle concentration up to 100 μg/ml had no significant effect on MG-63 cell viability. Sui *et al*. developed PLLA/HA hybrid membranes *via* electrospinning of a PLLA/HA dispersion. The authors found that the inclusion of HA nanoparticles implied a considerably increment in the roughness and in the mechanical properties of the matrix, as well as a decrease in its degradation rate in water. MG-63 osteoblast-like cells were cultured on PLLA and PLLA/HA membranes, finding a higher cell adhesion and growth in the latter substrates. A three-dimensional matrix was introduced by Zhang and co-workers, that described the fabrication of PLGA hollow fiber membranes using a wet phase-inversion approach and doped with HA nanoparticles. The aim was to obtain an aligned, bioactive and biodegradable scaffold mimicking the natural histological structure of human long bone, and they obtained an anisotropic membrane with a rough outer skin and a smooth inner skin (Fig. 6(A)). The structure showed a highly porous morphology with a unique asymmetric finger-like macroviod structure ideal to promote neovascularisation (Fig. 6(B)). pH values of the culture media at different time-points were evaluated for pure PLGA membranes and PLGA/HA membranes, finding that they were closer to neutral for the composite matrices; nanoHA powders in the composite structures may facilitate to neutralize the culture media. The bioactivity of such 3D scaffolds was also evaluated, by immersing them in SBF for several days, finding a significantly higher biomineralization rate for the nanodoped matrix in comparison with the pure PLGA scaffold. Finally, Torres *et al*. tested the mechanical properties and bioactivity of porous PLGA/TiO_2 nanoparticle-filled foams produced by thermally induced solid-liquid phase separation (TIPS) and subsequent solvent sublimation. By manipulating the parameters of the TIPS process, they were able to control the porous architecture, determining the presence of orientated tubular macropores (Fig. 6(C), (D), and (E)). The bioactivity of the scaffolds was also demonstrated by immersion in SBF for up to 28 days, and confirming the formation of hydroxyapatite crystals on the scaffold surface.

The aim of doped biomaterials is not always to promote an enhancement of cell adhesion and proliferation; in some cases, a specific application requires the inhibition of the activity of a certain cell population. In the central nervous system (CNS), astroglial cells proliferate and produce an ECM protein-rich glial scar to isolate any implant (for example a neural electrode) and, in general, regeneration after injury in the CNS is inhibited primarily due to astrocytic glial scar formation. It was therefore proposed a novel composite material made of polyurethane (PU) and doped with zinc oxide (ZnO) nanoparticles as nerve guidance channel material able to significantly reduce astroglial cell adhesion and proliferation (Seil & Webster, 2008). Importantly, ZnO nanoparticles possess piezoelectric properties, that make them promising for neural regeneration applications. Regarding astrocyte behaviour on such materials, the collected data showed a reduced ability of these cells to adhere and proliferate on ZnO nanoparticle PU composites with higher nanoparticle concentrations. The authors finally proposed that the piezoelectric properties of such composites may provide the stimulatory cues necessary to promote neural cell function.

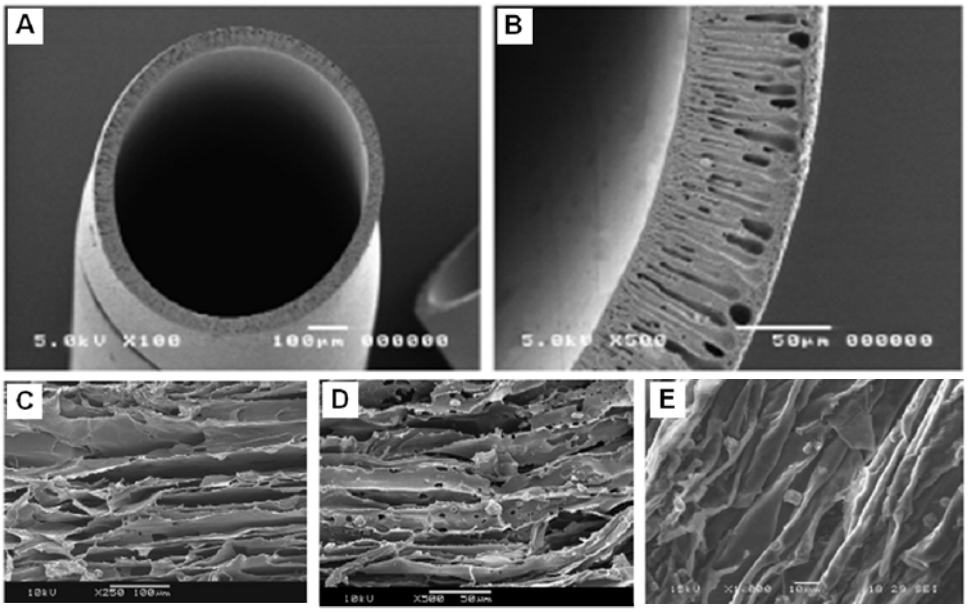

Fig. 6. (A), (B): PLGA hollow fiber membrane doped with 30% nanoHA; (C), (D), and (E): foam scaffolds made of plain PLGA(C), PLGA + 5 wt% TiO_2 (D), and PLGA + 20 wt% TiO_2 (E). Some TiO_2 agglomerates are visible in the 20 wt% foam. Images from (Zhang *et al*., 2007) and (Torres *et al*., 2007). Reproduced with permission from Elsevier

In the same year, several studies regarding bone tissue engineering were published (Erisken *et al*., 2008-b; Guan *et al*., 2008; Nejati *et al*., 2008; Schneider *et al*., 2008; Duan *et al*., 2008; Wei *et al*., 2008). An interesting PHB-based scaffold containing nanosized hydroxyapatite was developed by Guan and co-workers by gas-jet/electrospinning. Bone marrow stroma cells (BMSCs) were tested on the material, finding that both proliferation rate and alkaline phosphatase (ALP) activity increased were higher on the nHAP/PHB scaffolds surface than on standard tissue culture plates and pure PHB scaffolds surface. Rod-shaped nano-hydroxyapatite and nHAP/PLA composite scaffolds were developed by Nejati *et al*. The rod shaped nHAP particles (37-65 nm in width and 100-400 nm in length) were similar to natural bone apatite in terms of chemical composition and structural morphology and they were used to build PLA nanocomposites using thermally induced phase separation method (Fig. 7(H), and (I)). Such scaffolds were found to be comparable with cancellous bone in terms of microstructure and mechanical strength, making them suitable for bone tissue engineering applications. Rat mesenchymal stem cells (MSCs) were tested on the material, finding that nHAP/PLA scaffolds appeared biocompatible and non-cytotoxic to the cells. Furthermore, after seven days of culture, round shape cells were found on the surface of pure PLA scaffolds (Fig. 7(L)), while cells on the nanocomposite scaffolds exhibited spindle shaped morphology and migrated through the pores (Fig. 7(M)). A flexible, cotton wool-like PLGA/amorphous tricalcium phosphate (ATCP) nanocomposite was prepared by Schneider and co-workers by electrospinning. It was shown that ATCP nanoparticles clearly influenced the fiber morphology (Fig. 7(A), (B), (C), and (D)), also enhancing the

biomineralization of the scaffold after immersion in SBF (Fig. 7(E), and (F)). Human mesenchymal stem cells (hMSCs) were cultured on the composite matrix (Fig. 7(G)), finding a good proliferation rate and an enhanced production of ALP and osteocalcin in comparison with the controls.

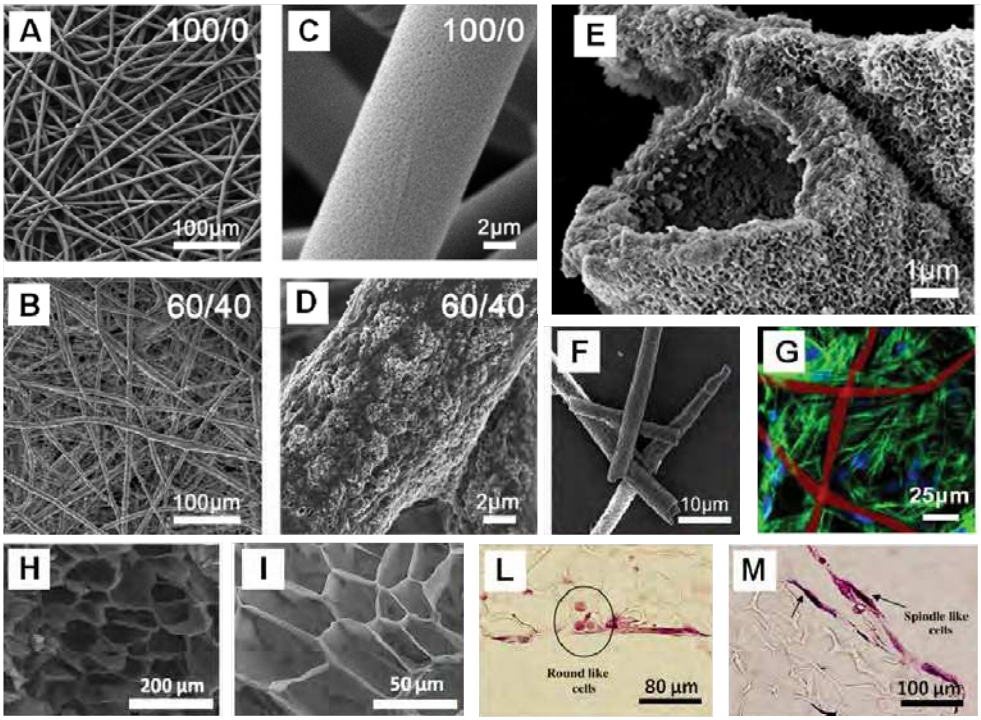

Fig. 7. (A), (C): SEM images of electrospun pure PLGA scaffold; (B), (D): SEM images of electrospun PLGA/ATCP (60/40) nanocomposite; (E), (F): SEM image of extracted PLGA/ATCP (60/40) tubes after 45h of immersion in SBF. The high-magnification image reveals a wall thickness of ~ 1 µm; (G): hMSCs morphology after 4 weeks of culture on the nanocomposite PLGA/ATCP scaffold. In blue: cell nuclei, in green: cytoskeletal actin, in red: scaffold architecture; (H): SEM image of pure PLA scaffold; (I): SEM image of nHAP/PLA composite scaffold; (L): optical microscope photographs of MSCs attached to the pure PLA scaffold; (M): optical microscope photographs of MSCs attached to the nHAP/PLA scaffold. Images from (Schneider *et al*., 2008) and (Nejati *et al*., 2008). Reproduced with permission from John Wiley and Sons and Elsevier.

A functionally graded non-woven mesh of polycaprolactone incorporated with tricalcium phosphate nanoparticles was fabricated by Erisken *et al*. using a new hybrid twin-screw extrusion/electrospinning process. With such method, the concentration of nanoparticles could be tailored in a targeted manner, changing the scaffold properties. MC3T3-E1 mouse preosteoblast cells were cultured on the matrix, showing a good proliferation and well differentiating into osteoblasts, converting the PCL-β-TCP non-woven composite meshes into a bone tissue-like constructs. Duan and collegues used the selective laser sintering

technology to produce Ca-P nanoparticle filled poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) microspheres, as bioresorbable osteoconductive composite scaffolds. Ca-P particles had a plate-like morphology with about 10 μm in length and approximately several nanometers in thickness; the composite matrix was prepared by means of emulsion solvent evaporation method. Neither *in vitro* and *in vivo* experiments were performed, but the authors foresaw that, once inside the human body, the Ca-P particles superficially embedded on the microspheres could promote the formation of bone-like apatite and enhance bone formation, while the internal Ca-P particles could stiffen the composite and gradually release calcium and phosphate ions with the degradation on the PHBV matrix. Wei *et al*. used the solvent casting methods to fabricate PDLLA films with 0 and 20 wt% TiO_2 nanoparticles and with 20 wt% TiO_2 mixed with 5 wt% micrometer-sized Bioglass® particles. *In vitro* bioactivity studies were carried out using SBF and culturing osteoblast-like MG-63 cells. The PDLLS films containing different concentrations of TiO_2 and Bioglass® particulate inclusions showed no effect on cell viability after 7 days of incubation, and they showed promising properties of bioactivity, making them attractive for bone tissue engineering.

A novel injectable scaffold for tissue engineering was developed in 2009 by Huang and collegues (Huang *et al*., 2009). The opportunity to use an injectable scaffold is attractive for *in vivo* perspectives, as it minimizes patient discomfort, risk of infection, scar formation and the cost of treatment. The developed substrate was made of nano-hydroxyapatite/collagen (nHAC) loaded on chitosan/β-glycerophosphate matrix. Bone-marrow-derived mesenchymal stem cells were tested, evaluating their proliferation capability. The composite material showed no toxicity and it did not interfere with the proliferation capability of MSCs, making it an interesting solution for bone tissue engineering, above all for its injectability (ability to flow out totally from a syringe).

Another matrix for bone regeneration was fabricated by Thein-Han and co-workers (Thein-Han *et al*., 2009), using a silicone rubber doped with nanohydroxyapatite and a process involving uniform dispersion of nHA *via* shear mixing and ultrasonication, followed by compounding at sub-ambient temperature and high-pressure solidification when the final curing reaction occurred. MC3T3-E1 mouse pre-osteoblast cells were cultured on the scaffold, finding that the cell density on the composite matrix was significantly higher than that on the pure silicone rubber. Furthermore, immunofluorescence staining revealed that cells on the composite matrix showed larger vinculin focal contacts with higher expression level at the edges, in comparison with the non-doped material. Cytoskeletal actin organization was also more prominent on the composite scaffold.

Regarding liver tissue engineering, titania/chitosan composite scaffolds were prepared by Zhao *et al*. through a freeze-drying technique (Zhao *et al*., 2009). Doped matrices with 0.3 of TiO_2/chitosan weight ratio showed the best mechanical performances, maintaining an interconnected pore structure. Hepatic immortal cell line HL-7702 was tested on these structures, detecting liver-specific functions, such as albumin secretion and urea synthesis. No differences were found between the doped scaffold and the pure chitosan matrix, in terms of albumin and urea production; FESEM micrographs of cells cultured on the different scaffolds revealed an better sustained attachment of hepatocytes on the doped matrix, probably thanks to the improved mechanical characteristics, implying the potential application of this material in liver tissue engineering.

Single-walled carbon nanotubes (SWNTs) are unique in structure and function, and they have recently received significant attention due to their potential to create nanostructured conductive materials. A composite SWNT/collagen material was recently proposed as

conductive peripheral nerve regeneration matrix (Tosun & McFetridge, 2010). Neuron-like PC12 cells were tested on collagen and SWNT/collagen scaffolds, finding an enhanced proliferation rate on the doped matrices, and a normal expression of p53 gene, indicating the absence of nanoparticle-induced DNA damage.

In another study, the influence of nanodoped matrices on cardiac and mesenchymal stem cells (CSCs and MSCs) was evaluated, using PLGA films loaded with TiO_2 and CeO_2 nanoparticles (Mandoli *et al.*, 2010). The hybrid nanoCeO_2/PLGA scaffolds showed a parallel surface pattern, with a spacing of about 250 μm between the ridges (Fig. 8(A)). Better CSC and MSC proliferate activity was observed for CeO_2 composites with respect to either TiO_2-added on unfilled PLGA films (Fig. 8(E)), together with a clear cell alignment in the direction of the ridges (Fig. 8(B), (C), and (D)).

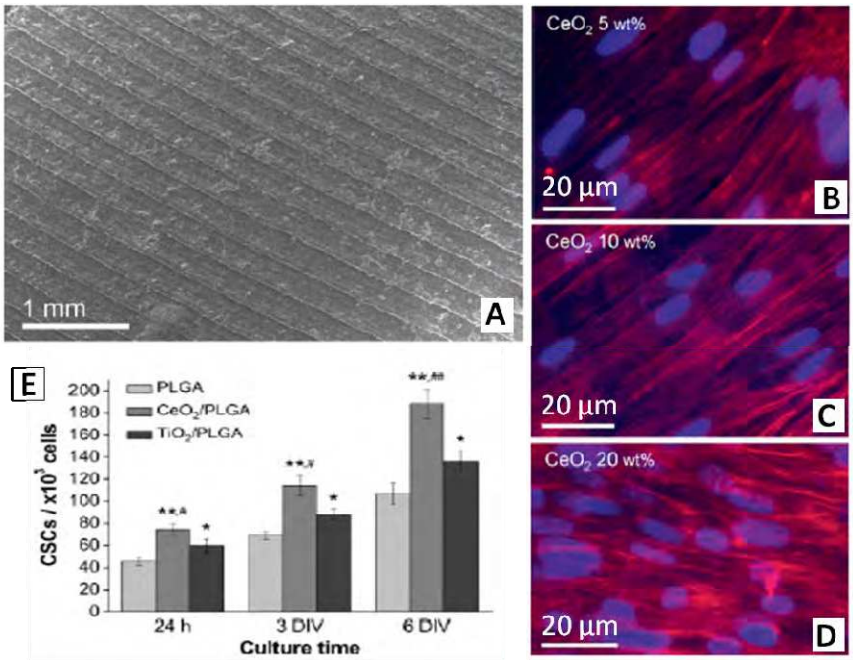

Fig. 8. (A): SEM image of parallel-organized 20% CeO_2/PLGA composite; (B), (C), and (D): CSC seeded on the composite substrates having different nanoparticle concentrations; (E): proliferation assay for the CSC culture on the different scaffolds (pure PLGA, CeO_2/PLGA and TiO_2/PLGA). Images from (Mandoli *et al.*, 2010). Reproduced with permission from John Wiley and Sons.

Skeletal muscle tissue engineering was also addressed using a nanodoped matrix-based approach (McKeon-Fischer & Freeman, 2010). PLLA and gold (Au) nanoparticles were electrospun to create three composite scaffolds: 7% Au-PLLA, 13% Au-PLLA, and 21% Au-PLLA. Rat primary skeletal muscle cells were cultured on the three scaffolds and on the control (pure PLLA matrix), finding that they showed low proliferation capability on the doped scaffolds. However, as demonstrated by the authors, this was not due to the

nanoparticle toxicity, but to an enhanced cell fusion into myotubes and a higher level of cell differentiation. The electrical conductivity of the scaffolds (increasing with the increase of nanoparticle concentration) and the good cell behavior on them are promising for skeletal muscle regeneration.

More recently, H9c2 rat cardiomyocytes were cultured on PLGA scaffolds loaded with different concentrations of barium titanate nanoparticles (BTNPs) (Ciofani *et al*., 2011). BNTPs have previously proven to be non-toxic even at high concentrations, and they have intriguing properties, related to their piezoelectric nature that could influence some cell activities. The PLGA/BTNPs were found to have increased mechanical properties in comparison with bare PLGA scaffolds, with a Young's modulus linearly increasing with the BNTPs concentration and the surface roughness was also increasing due to the nanoparticle presence. H9c2 cells showed an increased proliferation on the doped matrices, probably related to the different protein adsorption due to the different surface roughness. The authors hypothesize that BNTPs piezoelectricity could also have a role in such cell behavior, also envisioning future nanoparticle-mediated scaffold stimulation.

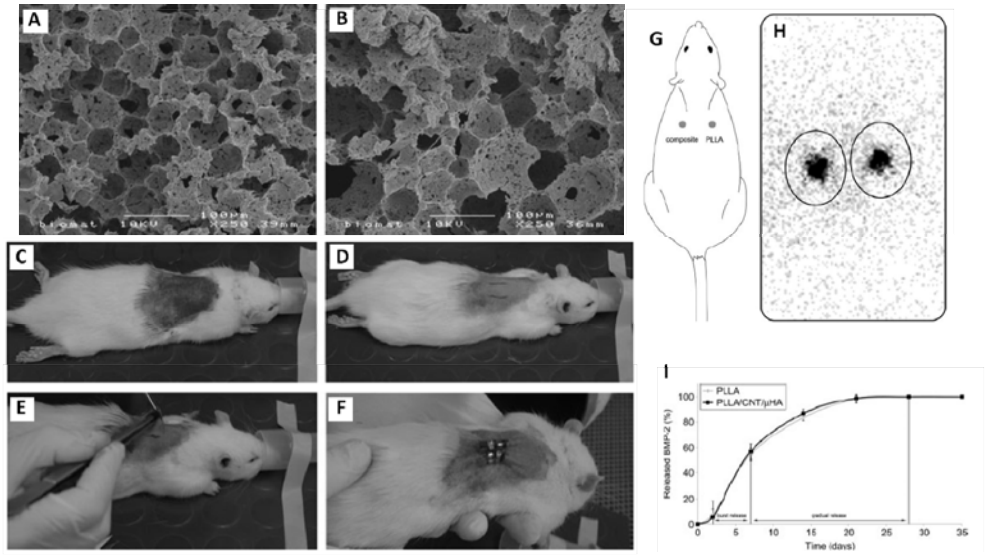

Fig. 9. (A) and (B): SEM images of PLLA and PLLA-CNT-μHA, respectively; (C), (D), (E), and (F): Overview of the subcutaneous implantation of the scaffolds on the back of the rat; (G): schematic overview of the scaffold placement; (H): scintigraphic image of the *in vivo* measurement of released ^{131}I-labelled BMP-2 in rat 14 days after implantation; (I): *in vivo* release profile of ^{131}I-labelled BMP-2 from the scaffolds. Images from (van der Zande *et al*., 2011). Reproduced with permission from John Wiley and Sons.

An interesting nanocomposite for wound dressing applications was fabricated using β-chitin and silver nanoparticles (Kumar *et al*., 2010). Such scaffold showed high porosity, which increased for the composite matrix. This is due to the inclusion of the nanosilver solution with the original β-chitin hydrogel; when the sample was freeze-dried, the water in the silver colloid, which got incorporated into the hydrogel, evaporated and the vacant

space was left as pores in the scaffold, ultimately resulting in a highly porous composite. Antibacterial studies revealed higher susceptibility of Gram-negative bacteria to nanosilver, with a bacteria inhibition zone larger for higher nanosilver concentrations. The scaffolds were also demonstrated to be cell-friendly, without significant toxicity induced on cultured cells. The β-chitin/nanosilver composites were therefore proposed as novel and promising wound dressing material.

A promising *in vivo* study was carried out by van der Zande and co-workers (van der Zande *et al*., 2011), that fabricated pure PLLA scaffolds (Fig. 9(A)) provided with bone morphogenetic protein-2 (BMP-2), to improve bone response. The problem to employ such material *in vivo* is that BMP-2 is clearly almost immediately released from the site of implantation by diffusion; to prolonge the retention of BMP-2 into the scaffold, the authors fabricated PLLA matrices doped with carbon nanotubes (CNTs) and microhydroxyapatite (μHA) (Fig. 9(B)).

Radiolabelled BMP-2 was loaded onto plain PLLA and composite PLLA-CNT-μ HA matrices, and the scaffolds were implanted subcutaneously for 5 weeks in rats (Fig. 9(C), (D), (D), and (E)). In contrast with authors' hypothesis, the *in vivo* release showed no differences between the composite and plain PLLA scaffolds. Nevertheless, although incorporated CNTs and μ HA in a PLLA scaffold did not alter the release pattern of BMP-2, they could still provide composite scaffolds with several other beneficial characteristics, as higher mechanical performances and enhanced bone formation.

5. Conclusion

The development of innovative materials useful as scaffolds for the sustained growth of cells is of particular interest in regenerative medicine and tissue engineering, because they can be potentially tailored to mimic the natural extracellular matrix in terms of structure, chemical composition, and mechanical properties.

In the past decades, an urgent necessity of innovative, "smart" materials for tissue engineering raised, in order to obtain constructs able to sustain and promote cell proliferation and tissue regeneration, and particular attention was dedicated to polymer/ceramics composites, in order to take advantage by the physico-chemical properties of these two classes of materials.

Nanostructured materials have been extensively explored in many biological applications because of their intriguing physical and chemical properties. In particular, the intrinsic optical, magnetic, and electrical properties owned by nanomaterials can offer remarkable opportunities of interaction with complex biological processes for several biomedical applications.

The advent of nanotechnology is set to accelerate development of improved and sophisticated smart material technologies. Researchers are now considering the possibilities of designing, altering, and controlling material structure at nanoscale levels in order to enhance material performance and process efficacy. The advancements in nanomaterials are expected to increase product quality and performance, and they are finding acceptance in diverse applications such as sensors and electronic devices. Nanosensor particles assist in creating tools for analyzing living cells and serve as reporters in industrial process monitoring. In the future, smart materials are likely to derive their success from nanotechnology that is likely to be instrumental in creating more varied, complex, and intelligent systems.

The advances and improvements in smart materials allow them to cater to a diverse set of applications, especially in the defense, aerospace, healthcare, electronics, and semiconductor industries. Although very few of these applications are at present commercially viable, their potential for future acceptance is enormous. Smart materials are particularly useful for cellular production: with the addition of cellular fluid and by regulating the cell's shape and mechanical conditions, smart materials can mimic these cells' interactions and exhibit effective results.

In our opinion, the combination of smart and nanomaterials, and the exploitation of their impressive chemical and physical properties, will constitute a fundamental step towards realistic clinical applications of tissue engineering and regenerative medicine.

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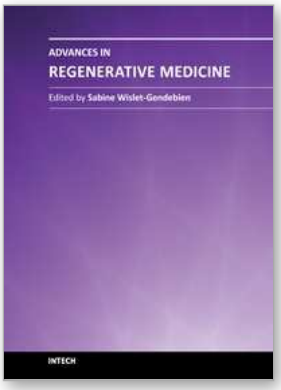

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Even if the origins of regenerative medicine can be found in Greek mythology, as attested by the story of Prometheus, the Greek god whose immortal liver was feasted on day after day by Zeus' eagle; many challenges persist in order to successfully regenerate lost cells, tissues or organs and rebuild all connections and functions. In this book, we will cover a few aspects of regenerative medicine highlighting major advances and remaining challenges in cellular therapy and tissue/organ engineering.

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