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Processing of Materials for Regenerative Medicine Using Supercritical Fluid Technology

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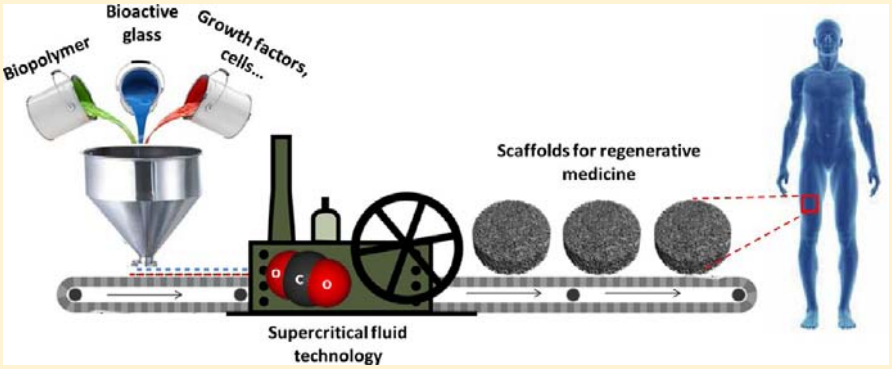

ABSTRACT: The increase in the world demand of bone and cartilage replacement therapies urges the development of advanced synthetic scaffolds for regenerative purposes, not only providing mechanical support for tissue formation, but also promoting and guiding the tissue growth. Conventional manufacturing techniques have severe restrictions for designing these upgraded scaffolds, namely, regarding the use of organic solvents, shearing forces, and high operating temperatures. In this context, the use of supercritical fluid technology has emerged as an attractive solution to design solvent-free scaffolds and ingredients for scaffolds under mild processing conditions. The state-of-the-art on the technological endeavors for scaffold production using supercritical fluids is presented in this work with a critical review on the key processing parameters as well as the main advantages and limitations of each technique. A special stress is focused on the strategies suitable for the incorporation of bioactive agents (drugs, bioactive glasses, and growth factors) and the *in vitro* and *in vivo* performance of supercritical CO_2-processed scaffolds.

1. INTRODUCTION: STATE-OF-THE-ART AND CHALLENGES OF MATERIALS FOR REGENERATIVE MEDICINE

Changes in the population pyramid are being experienced worldwide in recent decades, leading to a new lifestyle paradigm as well as new social and health needs. In Europe, the number of people older than 65 years is projected to dramatically rise from 92 million in 2013 toward 152 million in the 2060 horizon.[1] The increase in life expectancy dictates the design of efficient and durable sanitary approaches to keeping older people active and independent longer, while allowing younger people access to early diagnosis and prophylactic action and trying to stop the current exponential growth in health care costs. In 2011, direct (hospitalization) and indirect (sick leave and in-home health care) health costs already represented around 8% of the GDP for the Member States of the European Union.[1,2]

The propensity for osteodegenerative diseases and accidental fractures is a relevant health concern not only in the elderly, but also in other age sectors. The popularization of sports practice by nontrained people also increases the incidence of musculoskeletal injuries, defects, and fractures with potential occurrence of severe pain. Bone grafts are needed in cases of large defects or osseous congenital deformities where spontaneous regeneration with conventional therapy is not possible. Every year, more than 2.2 million people worldwide require bone grafting surgical procedures.[1] Nevertheless, autologous and donor grafts are limited and not exempt from clinical complications such as slow or deficient recovery of the transplanted bone region or occurrence of infection and inflammatory response.[3,4] Alternatively, non-osteoinductive synthetic materials (made of metal, calcium phosphate ceramics, or bioglasses) have short durability and may be deficiently integrated in the host bone or even rejected. This scenario has prompted an intensification in the design and development of an upgraded generation of synthetic implants acting as biodegradable 3D-constructs with tailored properties. These new grafts should not only act as temporary physical templates (scaffolds) for tissue regeneration, but also play an active role in guiding and promoting tissue growth or even in

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delivering bioactive substances in an adequate sequence in the implant zone. Advances in regenerative medicine are the result of intense interdisciplinary collaboration between scientists of various fields and clinicians who search for stimulation of the body's own self-healing capacity.[5] The implants used for these purposes are usually the result of a synergistic combination of biocompatible matrices (scaffolds), living cells, and/or bioactive compounds, as follows.

- Biocompatible matrix is the component most susceptible to be engineered to mimic the physiological function of the ECM with the purpose of serving as mechanical support and template for tissue growth and of preserving the cells' ability to differentiate. The matrix should show biological acceptance, biodegradation rate compatible with the tissue growth rate, and suitable textural properties to facilitate cell ingrowth, adhesion, proliferation, and reorganization, as well as neovascularization and diffusion of nutrients and gases to cells. Regarding the porous structure, an ideal matrix should exhibit a highly open and uniform porosity (>80%) with nanopores (micro- or mesopores, for cell attachment) and macropores (>100 μm, for vascularization and bone ingrowth) interconnected between them (ca. 100%, to supply nutrients and oxygen to the cells and to eliminate cell wastes). Moreover, the biocompatible matrix should be capable of withstanding the mechanical stresses exerted on the scaffold (compressive modulus of ca. 100 kPa for bone temporary scaffolds[3]), which are inherent in the bone function.
- Living cells from adult stem cells can be harvested *in vitro* and then incorporated in the biocompatible matrix to form the tissue or can be attracted *in vivo*. Cells will have to develop microvasculature and microcirculation in the synthetic bone construct, which is needed for the transport of oxygen, nutrients, and soluble factors that are crucial for bone regeneration and homeostasis.[4]
- Bioactive compounds in the form of growth and differentiation factors can interact with the cells before implantation (i.e., *in vitro*) or, in a more efficient approach, be incorporated in the scaffold and interact with the cells after implantation (i.e., *in vivo*). The main role of these bioactive compounds is to regulate cell proliferation, differentiation, migration, motility, and adhesion in the growing tissue. The binding of the bioactive factors to the scaffold is crucial to get release profiles that can mimic the natural sequences of tissue morphogenesis or regeneration, avoiding potential toxicity at systemic levels.[5]

Many innovative manufacturing solutions for the production of synthetic implants for regenerative medicine are being currently engineered under different stages of development. Implant production can be classified in different ways according to material origin (natural, semisynthetic, or synthetic polymers), processing approach (bottom-up or top-down), and manufacturing principle (solvent drying, phase separation, fusion, leaching, or additive manufacturing). According to the latter classification criteria, several processing techniques have been developed to manufacture 3D-scaffolds, namely, solvent casting + particle leaching, freeze−drying + particle leaching, thermally induced phase separation, immersion precipitation, laser sintering, compression molding, injection molding, extrusion, foaming, and electrospinning.[3,6] Although somewhat successful, these conventional techniques have several drawbacks for scale-up, especially concerning the use of organic solvents, vigorous mechanical agitation, and heating. Furthermore, simultaneous control of macro- and microstructure of the scaffold is difficult. In past years, processing with supercritical fluids, mainly $scCO_2$, has been extensively evaluated.[7] The special physicochemical properties of this innocuous fluid allows the processing of polymers and inorganic materials at mild operating conditions, avoiding or mitigating the use of organic solvents.

This article provides an overview of the state-of-the-art in supercritical fluid technology for manufacture of scaffolds and intermediate components for regenerative medicine. Fundamentals and processing parameters of the different methods, materials manufacturing routes, and case studies with representative scaffolds from synthetic (mainly PLLA, $P_{D,L}A$, PLGA, and PCL) and natural (chitosan, silk fibroin) polymers and hybrids thereof will be outlined. Examples of incorporation of bioactive agents in the scaffold and results of *in vitro* and *in vivo* tests with cells will be analyzed.

2. SUPERCRITICAL FLUID TECHNOLOGY: $SCCO_2$ PROPERTIES AND MAIN PROCESSING TECHNIQUES

Supercritical and near-critical fluids, namely, $scCO_2$, have emerged as an attractive solution for regenerative medicine purposes and related fields such as pharmaceutical, food, and agrochemical technologies.[8] This kind of fluid allows the design of materials of different composition (organic, inorganic, or hybrid), morphology (micro- and nanoparticles, monoliths, beads, sponges), porosity (meso- and macro-porosity), and inner architecture (homogeneous, multicomponent, multilayered). Moreover, processing with supercritical and near-critical fluids operates under mild conditions and leads to a solvent-free end-product with high purity.

Fluids turn supercritical at temperature and pressure above those of the critical point (T_c and P_c, respectively). Supercritical fluids, mainly $scCO_2$, are endowed with unique physicochemical properties for materials processing. Change of diffusivity, viscosity, and density with operating pressure and temperature of supercritical fluids opens up the possibility of tuning the composition and morphology of the end material. Moreover, absence of surface tension enables supercritical fluids to totally wet materials with intricate morphologies and textures, including micro- and meso-porous substrates, without collapse of the nanostructure.

$scCO_2$ is the most employed supercritical fluid due to the mild conditions of its critical point (7.38 MPa, 304 K), which are adequate for labile materials. Moreover, it is nontoxic, nonflammable, cheap, and relatively inert, and referred to as GRAS. Because of the gaseous state of CO_2 under ambient conditions, a dry solvent-free product is obtained upon depressurization. $scCO_2$ can be applied for materials processing purposes following four different strategies, as explained below (Figure 1).[9]

1. $scCO_2$ as a Solvent.[10] Due to the absence of dipole molecule and the weak quadrupole moment of CO_2 molecule, $scCO_2$ is only able to solubilize organic molecules mainly with low hydrophilic/hydrophobic character, low molecular weight, and low polarity. Hydrocarbons and aromatic compounds and small molecules containing esters, ethers, silane, lactones, or epoxy groups are among the substances susceptible to be solubilized by $scCO_2$. Small amounts (5−10 wt %) of

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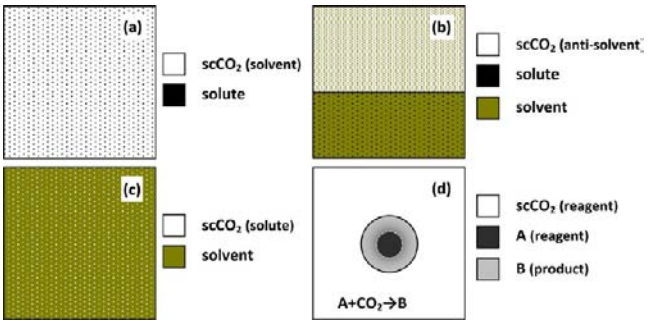

Figure 1. Different approaches for the application of supercritical fluid technology to materials processing, using $scCO_2$ as (a) a solvent, (b) an antisolvent, (c) a solute, and (d) a reagent. Reproduced from ref 9a with permission of the author.

cosolvents (e.g., acetone, ethanol) are typically mixed with $scCO_2$ in an attempt to improve the affinity of the resulting medium to polar molecules. This approach is used in the industry for extraction of essential oils, nutraceuticals, and fragrances from plants, particles formation (RESS process), and drying and impregnation of porous and nanostructured substrates, and as media for polymerization and enzymatic reactions.

2. $scCO_2$ as an Antisolvent.[11] Under this processing strategy, $scCO_2$ presents a partial or total miscibility with other solvents, but behaves as a nonsolvent for the solute. A two-way mass transfer phenomenon takes place: (i) CO_2 rapidly diffuses into the solution and (ii) the solvent dissolves into the nonsolvent ($scCO_2$ in this case). This causes the solvation power to decrease, and the subsequent supersaturation of the solute provokes its precipitation. GAS, SAS, PCA, ASES, and SEDS particle processing methods and preparation of scaffolds through supercritical fluid-assisted phase inversion (section 3.2) exploit $scCO_2$ as antisolvent. The outcome of the process can be tuned by the order of addition of the liquid and the $scCO_2$ into the precipitation vessel (sequential or simultaneous), the use of a restrictor (nozzle), and the flow regime (discontinuous, semicontinuous, or continuous).

3. $scCO_2$ as a Solute.[9b] The interest in $scCO_2$ as a porogenic agent is based on its capacity to dissolve in amorphous and semicrystalline polymers.[12] Pores are formed upon depressurization and subsequent release of the $scCO_2$ dissolved in the polymeric matrix (section 3.1). The plasticizing effect of $scCO_2$ reduces the melting point and glass transition of polymers and is being exploited for extrusion processes.[13] This strategy is also applied in the so-called PGSS process (section 4.2) for the preparation of polymeric particles. The technique consists of first dissolving $scCO_2$ in a molten solid at low operating temperatures followed by sudden expansion through a restriction. Complete evaporation of the CO_2 takes place upon expansion, leading to the solidification of the polymeric melt into solvent-free microparticles with high process yields.[14]

4. $scCO_2$ as a Reagent.[15] Industrial consumption of CO_2 for the synthesis of low-molecular-weight compounds is commonly hampered by the use of carbon monoxide (CO) as an alternative reagent. Despite CO being more toxic and representing a higher health and safety risk than CO_2, the former has higher reactivity. This problem could be overcome using $scCO_2$, which would increase the concentration of CO_2 in the reaction medium. The good diffusivity of $scCO_2$ might accelerate heterogeneous reactions involving porous solids with diffusion processes as a rate-controlling step. Finally, the use of $scCO_2$ as solvent and reagent at the same time can be envisaged for the synthesis of chemicals such as carbamates.

3. USE OF SUPERCRITICAL FLUIDS IN SCAFFOLD PREPARATION

3.1. Supercritical and Compressed CO_2 Foaming. Conventional foaming methods use organic solvents that may be harmful to the cells, growth factors, and surrounding living tissues if still remaining in the scaffold when implanted. Foaming using $scCO_2$ or compressed CO_2 as a solute of the polymer (see 3 in former section) enables precise control of the porosity and porous morphology and leads to solvent-free scaffolds upon depressurization. CO_2 removal caused thermodynamic instability due to supersaturation of CO_2 in the polymer matrix and generates gas/SCF nuclei in the bulk of the polymer. The nuclei grow upon further diffusion of CO_2 out from the polymer, resulting in the expansion of the polymeric matrix and the subsequent reduction in the polymer density. This foaming method is restricted to polymers fulfilling two criteria: (1) enough affinity to CO_2 sorption and (2) T_g values lower than the operating temperature (taking into account the T_g decrease by CO_2). The resulting material has a porosity that depends on the CO_2 sorption in the polymer during the foaming, which can be tuned by means of the temperature and pressure. The interconnectivity depends on the processing conditions, mainly the depressurization and cooling rates.[16] Enough soaking time is needed to reach the CO_2 sorption equilibrium; otherwise, lower CO_2 content in the polymer and a less uniform foam structure will be obtained.[17] The pressure drop upon depressurization and the cooling rate play a key role in pore formation, interfering in the competition between pore nucleation and growth.[18] The obtained scaffolds present a nonporous dense outer skin (Figure 2) due to the rapid

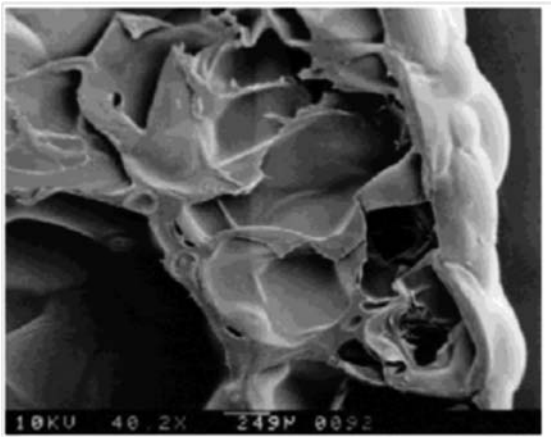

Figure 2. Typical nonporous external layer (right of the picture) of a polymer processed with compressed CO_2 or $scCO_2$ gas foaming. Reproduced from ref 20 with permission of John Wiley & Sons.

diffusion of the gas dissolved in the vicinity of the sample edges.[19] This skin may hinder cell migration to the inner part of the scaffold and should be removed before implantation.

Supercritical foaming for scaffold preparation was cited for first time in 1991 in the patent by De Ponti et al.[21] They prepared scaffolds from $P_{D,L}LA$ and PLGA solely or loaded with growth factors (GFs) to be used in surgery, therapy, or prophylaxis. The first scientific publication referred to the use of compressed CO_2 (5.5 MPa, rt, 72 h) foaming for biomedical

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applications dates from 1996 by Mooney et al.[19] The obtained solvent-free PLGA sponges had high porosities (94%), pore sizes of ca. 100 μm, and partial interconnectivity, but exhibited poor mechanical properties. Foaming of PLGA using other compressed gases (N_2, He) was attempted with unsuccessful results.[22] The effect of PLGA composition (L/G ratio and molecular weight) on compressed CO_2 foaming was tested. L/G ratios in the ranges 50:50 and 85:15 led to porosities greater than 90%. Lower molecular weights (i.e., lower intrinsic viscosities) of PLGA resulted in higher porosities, due to a lower resistance to expansion upon depressurization. An angiogenic factor, VEGF, was added to PLGA scaffolds during the foaming step resulting in incorporation yields of 72% and sustained release for 70 days in culture medium.[22]

$scCO_2$ foaming of PLGA (17.0−23.0 MPa, 308 K) significantly reduced the soaking time (0.5−2 h) with respect to compressed gas CO_2 foaming.[17,23] Scaffold pore size increased as the molecular weight of PLGA decreased up to 13−15 kDa, below which the scaffold became extremely fragile.[17] Lower L/G ratio led to scaffolds with smaller mean pore size and narrower pore size distribution.[17,24] More homogeneous structure and larger pore sizes were obtained for PLGA and $P_{D,L}LA$ scaffolds using lower depressurization rates, since these operating conditions decrease pore nucleation rate and promote pore growth and coalescence.[17,25]

Loadings of an inorganic phase into polymeric-based foams may improve the mechanical properties to withstand load-bearing applications as well as the bioactivity and biological behavior of the scaffold. Hydroxyapatite, bioactive silicate-based glasses, and phosphate-based glasses are being evaluated for these purposes. Filler composition and content influence the performance and biodegradation of the scaffold.[26] For example, $scCO_2$ foaming allowed the preparation of PLGA(75:25) scaffolds containing several bioactive ingredients (indomethacin, hydroxyapatite, catalase enzyme) at high loadings (20, 40, and 50 wt %, respectively).[23,24] The combined incorporation of hydroxyapatite and collagen to PLGA scaffolds during the supercritical foaming favors both the mechanical strength of the construct and the attachment of osteogenic cells.[27] Alternatively, a postprocessing method consisting of the infusion (applying pressure or centrifugation) of collagen into the pores of a hydroxyapatite-containing PLGA scaffold has been recently proposed.[28]

Scaffolds from semicrystalline PCL can also be obtained by supercritical foaming despite an existing reduction in CO_2 sorption and gas diffusivity with respect to PLGA. Pore nucleation in semicrystalline polymers is usually nonuniform, since the pores are generated through heterogeneous (at the interface between the remaining polymer crystallites and the amorphous phase) and homogeneous (in the amorphous phase) nucleations.[29] Heterogeneous nucleation is also promoted with the incorporation of particles (hydroxyapatite, talc, silica) to PCL prior to foaming.[30] In general, temperatures in the 303−313 K range are enough to melt PCL due to the melting point depletion effect of compressed CO_2.[31] Upon depressurization, the melting point does not increase at the same rate as the sorbed CO_2 content decreases, leading to PCL crystallization and subsequent rough microtexture rather than to the typical smooth surface obtained for amorphous polymers.[31c] In general, prolonged soaking and depressurization favor growth of pore sizes, whereas short cooling and depressurization times induce higher porosity and lower bulk density of the scaffold (Figure 3).[31b,c] PCL scaffolds with

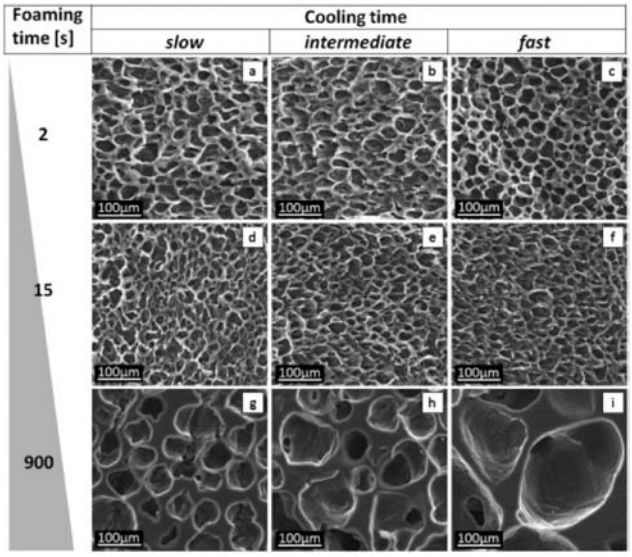

Figure 3. Porous morphology of PCL scaffolds processed by supercritical foaming. Effect of increasing cooling rate (from left to right) and depressurization rate (from bottom to top) on mean pore size and pore distribution. Reproduced from ref 30b with permission of Elsevier.

bimodal pore distribution and extensive interconnections have been obtained by using a two-step depressurization profile: (1) pressure decrease up to an intermediate pressure and, after a certain re-equilibration time, (2) depressurization to atmospheric pressure.[30b] Addition of cosolvents (ethanol, acetone, ethyl lactate, ethyl acetate) to CO_2 as blowing agents favors PCL plasticization and facilitates the supercritical foaming with more uniform pore size, although it might be incompatible with the incorporation of bioactive compounds.[32] Recently, PCL/porous SiO_2 NPs scaffolds were loaded with dexamethasone in two steps: first, the drug was loaded into the porous particles by supercritical impregnation, and then the PCL/dexamethasone-loaded SiO_2 was processed by supercritical foaming.[30a] Adjustable prolonged release can be obtained by changing the scaffold composition (SiO_2-to-PCL ratio) and the foaming operating conditions (pressure and depressurization rate).

Despite $scCO_2$ being a polymer plasticizer, high temperature (468 K) is generally required for melting and CO_2-saturation of PLLA (especially if the molecular weight is high).[33] Supercritical foaming of PLLA (468 K, 10.0−25.0 MPa, 10 min) gives rise to porous scaffolds with a pore density influenced by the depressurization rate and the saturation pressure as well as a tunable pore size and interconnectivity with the cooling rate.[16b] Lower CO_2 sorption in the biopolymer and a subsequent lower foaming extent of the scaffold is obtained at lower temperatures due to the crystallinity of PLLA homopolymer.[22] Nevertheless, compressed CO_2 foaming of PLLA and $P_{D,L}LA$ at ambient temperature should be considered for the incorporation of thermally labile species.[23,25,34] Georgiou et al.[26] have produced phosphate glass-containing PLLA scaffolds (porosity >75%; half of the pores with sizes between 200 and 400 μm) through a three-step processing method: melt-extrusion, vacuum drying, and supercritical CO_2 foaming (150−250 bar, 368 K). The inorganic filler led to less homogeneous structure, higher foam density, and higher T_g due to polymer/filler surface interactions, as also observed for PLLA-TCP[35] and PLLA-hydroxyapatite[36] composites. In general, the inorganic filler reduces proliferation of human primary osteoblasts, in

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comparison to a pure PLLA scaffold processed in a similar way, but induces higher differentiation into osteoblasts.[26,16b] $scCO_2$-processed PLLA scaffolds containing TCP or hydroxyapatite (up to 5%, the upper limit to obtain a suitable scaffold in terms of foam density, pore homogeneity, and interconnectivity[33a]) showed good biocompatibility and osteoconductive properties after 18-week implantation in rat cranium critical size defects.[37] In sheep, PLLA-TCP scaffolds (cylinders of 15 mm × 5 mm) showed good bone in-growth and absence of inflammation after one year of implantation in cancellous bone defects.[38] Finally, Oreffo and co-workers extensively proved the bone regenerative capacity of $scCO_2$-foamed $P_{D,L}LA$ scaffolds containing either VEGF[39] or BMP,[40] and both together[41] by seeding of hBMSC cells. In a different approach, chitosan/chondroitin sulfate NPs previously loaded with bioactive agents (BSA, platelet lysate) were shown to improve the homogeneous distribution of the agents into $P_{D,L}LA$ scaffolds (20.0 MPa, 308 K, 0.5 h) and their wettability, without significantly compromising their morphology.[34b,42]

Polymeric scaffolds containing hydrosoluble particles (e.g., salts, sugars, gelatin) can be subjected to postprocessing by leaching to yield macropores within the polymer matrix.[43] As an example (Figure 4), PLGA foams embedded with NaCl

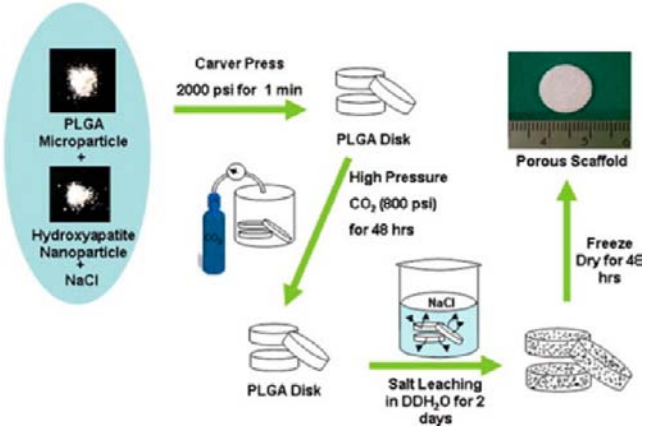

Figure 4. Preparation of PLGA + hydroxyapatite scaffolds: Sketch of the compressed CO_2 foaming plus particulate leaching process using NaCl as porogen salt. Adapted from ref 44 with permission of John Wiley and sons.

particles were produced by compressed CO_2 foaming (5.5 MPa, rt, 48 h), and then NaCl was leached out (H_2O, rt, 48 h).[20] Total porosity (85.1−96.5%), macroporosity, and pore interconnectivity could be tuned by the polymer/NaCl ratio (100:0 to 50:50) and the size of the salt particles (106 to 425 μm). Dual porosity was observed for these scaffolds: interconnected macropores coming from particulate leaching, and small and closed pores arising from the compressed gas foaming. Mechanical properties of the scaffolds (compressive and tensile moduli) were improved with respect to a scaffold of the same composition obtained by conventional solvent casting-plus-particulate leaching method (increase in 82% and 229%, respectively). Although no solid skin on the exterior surface of the foamed matrix was formed, smooth muscle cells only proliferated in the periphery of the scaffold. *In vivo* wettability and osteoconductivity of PLGA scaffolds can be enhanced by incorporation of calcium phosphate ceramics (100 nm diameter) to the polymer/leachable substance starting mixture (Figure 4).[44] Alternatively, after CO_2 foaming-plus-leaching the scaffolds can be exposed to gas (N_2, O_2) plasma treatment (100 W, 10 mTorr for 60 s) as a way to decrease water contact angle (from 79.2° to ca. 5° in the case of PCL scaffolds) and enhance mouse preosteoblast (MC3T3-E1) cell adhesion and proliferation.[45]

Supercritical foaming followed by leaching avoids the organic solvent required for standard solvent casting-plus-particulate leaching method, but still has the risk of discharge of bioactive agents during the leaching step.[22] Moreover, this technique needs long processing times and the final drying of the scaffolds. For example, PLGA scaffolds containing VEGF, alginate (to reduce VEGF release rate[22]), and NaCl particles (foamed at 5.9 MPa, rt, 20 h) only retained 44 ± 9% of the VEGF after leaching.[46] Nevertheless, the resulting scaffolds showed the growth of a bone-like apatitic layer on the inner pores after a 5 day incubation in SBF without an appreciable decrease of the initial total porosity (93%), and promoted the proliferation of hMVEC cells. VEGF release was sustained and localized (up to 2 cm distance from the implant), promoting vessel formation in the growing tissue.[22,46,47] Finally, pre-encapsulation of the GF in microspheres prior to foaming can give slower release rates at the expense of an additional processing step and further decrease in total VEGF payload.[46]

3.2. Supercritical Fluid-Assisted Phase Inversion.

Phase inversion or immersion−precipitation is a conventional process for preparing porous materials suitable for different biomedical applications.[48] In brief, this process involves the immersion of a mixture consisting of a polymer solution and, optionally, additives (e.g., bioactive agents) into a nonsolvent, thus inducing a thermodynamic instability and the subsequent precipitation of a polymer-rich phase.[49] Formation of pores and nanostructuration through interlinking of crystalline particles are promoted by fluid−fluid phase demixing and crystallization, respectively.[7a,49] The rates of these two processes can be tuned by changes in the operating parameters (polymer concentration, type of solvent) (Figure 5).[48,50]

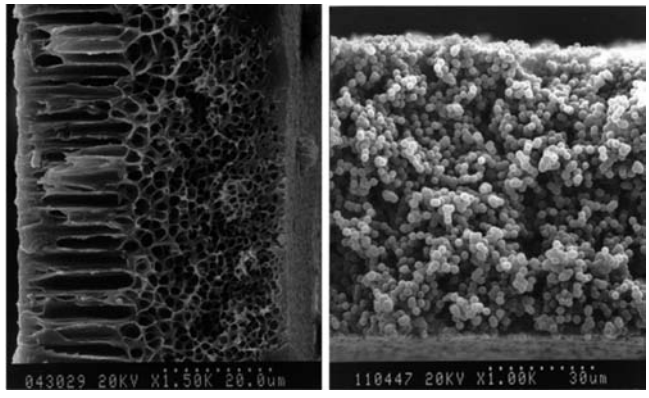

Figure 5. Porous polymeric membranes prepared by immersion precipitation. Formation of a cellular structure (left) and uniform microporous structure of spherical particles (right) can be obtained from the same polymer depending on the operating conditions. Reproduced from ref 50 with permission of Elsevier.

Supercritical fluid-assisted phase inversion involves the use of $scCO_2$ as the nonsolvent of the immersion−precipitation technique. In this case, operating temperature and pressure are the main parameters to tailor the properties of $scCO_2$ and the subsequent morphology and pore size of the material. Operating temperature, typically 318−328 K, along with the

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presence of organic solvents may be not suitable for the incorporation of GFs. After phase separation, flushing with $scCO_2$ is needed for removal of the solvent, which can also lead to the extraction of $scCO_2$-soluble compounds, e.g., polymer impurities or bioactive agents. Thereby, the incorporation yield of the bioactive agents may be low.[51]

Using this technique, PLLA scaffolds were prepared with controlled pore size distribution.[52] The average pore size decreased with the increase of CO_2 density (i.e., by decreasing the temperature or increasing the pressure) (Figure 6) and of

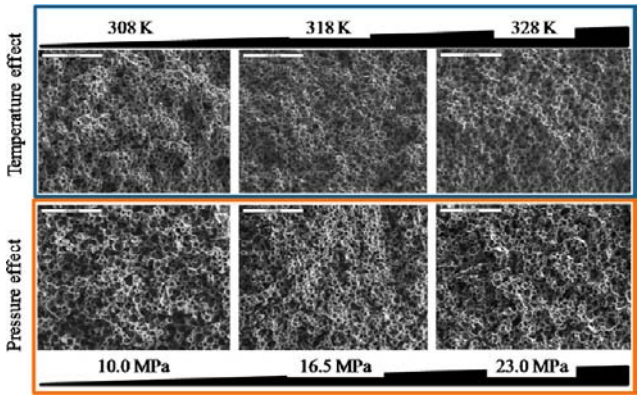

Figure 6. Preparation of PLLA scaffolds by supercritical fluid-assisted phase inversion. Effect of operating temperature (top) and pressure (bottom) on the porous structure of the material. Scale bar: 500 μm. Adapted from ref 52 with permission of Elsevier.

the initial polymer concentration in the dichloromethane solution. Fluid–fluid demixing, the kinetically favored mechanism, prevailed against polymer crystallization, the thermodynamically favored mechanism. Scaffold properties (pore size and pore density) were influenced by the addition and dispersibility of fillers for foam reinforcement (e.g., montmorillonite), which induce heterogeneous nucleation.[33b,53] In a different approach, highly porous scaffolds with both large pores and micropores were obtained by incorporating a solid porogen (e.g., ammonium bicarbonate) into the scaffold through phase inversion followed by heating above the decomposition temperature of the porogen (309 K).

The phase inversion technique has been also applied to prepare scaffolds from natural polymers.[54] Chitosan scaffolds produced using acidified water as solvent and $scCO_2$ + ethanol as nonsolvent were cytocompatible and allowed hMSC adhesion and proliferation.[54b] Scaffolds from natural polymer-containing blends (chitosan-PLLA, starch-PCL, and starch-PLLA) and composites (starch-PCL-bioglass) may combine the physicochemical and biological properties of the natural polymer with the mechanical properties and ease of processing of synthetic polymers.[55] After choosing the proper solvent able to dissolve both polymers, the solution undergoes $scCO_2$-based phase inversion leading to homogeneous structures with porosities up to 88% and mean pore sizes of ca. 75 μm with potential to be used in regenerative medicine.

3.3. Supercritical Drying of Gels. Sc-drying of certain organogels is particularly useful for obtaining aerogels, namely, lightweight materials with outstanding surface area (1000 m^2/g) and open porosity (95–99%) that preserve the original porous texture of wet gels. The presence of supercritical fluid mixtures in the gel pores avoids any vapor–liquid transition and surface tension and prevents the pore collapse. Sc-drying is a time-dependent two-way mass transfer of $scCO_2$ and gel solvent to and from the pores of the wet gel governed by several phenomena (liquid swelling and spillage, convective flow, and diffusion) (Figure 7).[56] Aerogels can be obtained

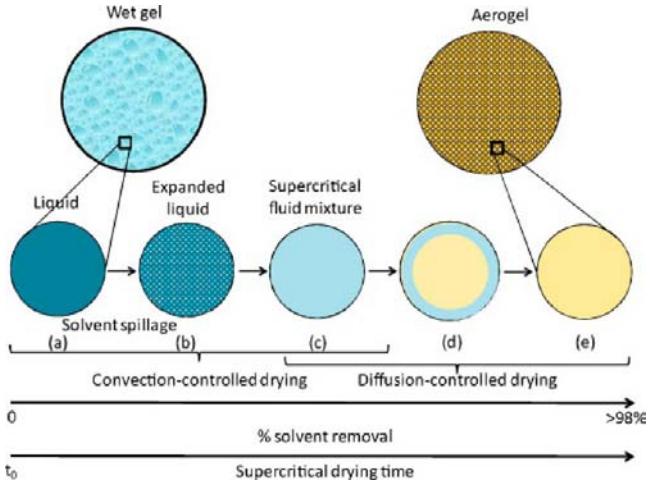

Figure 7. Supercritical drying profile of wet gels (intake corresponds to gel pores). The liquid content in the pore evolves from (a) gel solvent to (b) expanded liquid and (c) supercritical fluid, and then evaporation occurs (d) from the inner region to the pore walls, until (e) complete removal leads to a dried pore. Reproduced from ref 56 with permission of Elsevier.

from different sources (synthetic polymers—PLLA, polyurethane; inorganic—silica, titania; natural polymers—polysaccharides, proteins; and hybrids) and with a variety of morphologies (microparticles, beads, and monoliths) suitable to face up to diverse biomedical demands.[57]

Aerogels are mainly mesoporous and need some templating strategy to generate macropores and to tune the pore size distribution, such as through particulate leaching, positive molds, negative molds, simultaneous gas foaming + sc-drying, or emulsion templating (Figure 8).[57g,58] Preshaped PLLA aerogel scaffolds with high porosity (96–97%), surface area (45 m^2/g), and interconnectivity (ca. 100%) were obtained by sc-drying (4 h) of PLLA gels (in dioxane/ethanol) followed by particulate (D-fructose) leaching with water.[58a] Pore nanostructure was formed by PLLA fibers (50 to 500 nm) providing roughness that promoted uniform cell attachment and growth (Figure 8c,d). Using the same technique, PLLA/hydroxyapatite (<200 nm) aerogel scaffolds showed improved biomimetism and mechanical properties (compressive modulus up to 123 kPa).[59] Finally, incorporation of GFs or cells into aerogels is not evident using this technique since the solvents that can be extracted from the gels using sc-drying are typically ethanol or acetone.

Regarding natural polymers, sc-drying of chitosan/hydroxyapatite gels in acetone led to aerogel scaffolds with high porosity (91%) and good mechanical properties (compressive strength of 151 kPa) without the need of a porogen, although with restricted control on macroporosity.[60] These scaffolds promoted cell attachment and proliferation.[60c] Silk aerogel scaffolds produced by sc-drying + particulate leaching showed good attachment of human fibrobast cells.[61] Starch aerogels were prepared through emulsion templating for the generation of macropores (Figure 8a). Cooling of oil-in-corn starch solution emulsions led to the entrapment of oil droplets within

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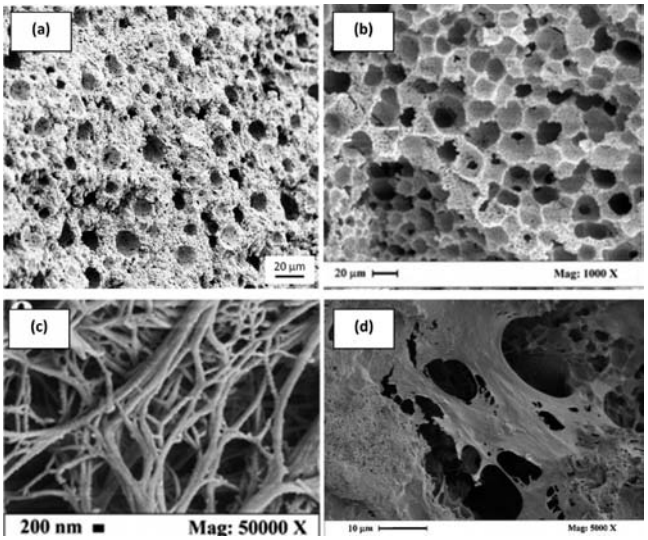

Figure 8. Generation of macropores in aerogels: (a) Macroporous structure of a starch aerogel scaffold obtained by emulsion-templating + supercritical drying (original photograph taken by the authors of this review). PLLA aerogel scaffold induced by supercritical drying + particulate leaching: (b) macroporous structure, (c) nanostructure, and (d) colonization of by hMSCs of the PLLA aerogel scaffold. Adapted from refs 7a and 58a with permission of Elsevier.

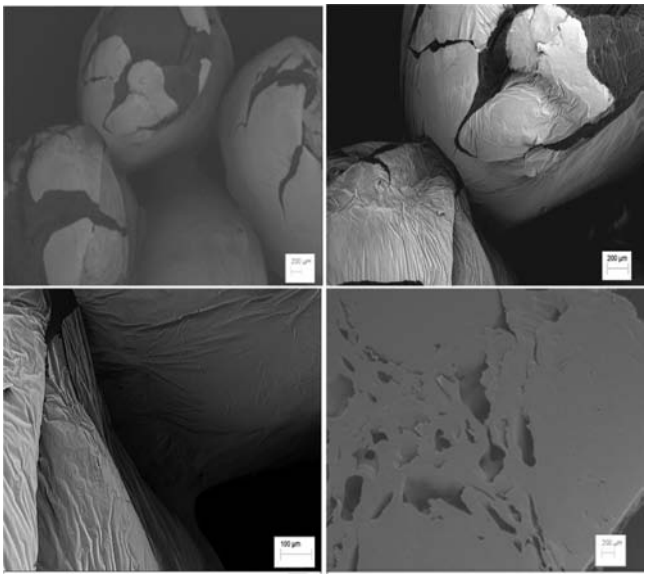

Figure 9. Sintering of PCL particles using $scCO_2$ (7.5 MPa, 310 K): (a,b,c) Short soaking times (1 min) led to the sintering of adjoining particles through plasticization of the surface; (d) longer soaking times (30 min) results in an incipient oversintering (original photographs taken by the authors of this review).

the gel. During sc-drying (313 K, 12.0 MPa, 8 h), not only the solvent of the gel, but also the remaining oil droplets were extracted, thus generating mesoporosity (4 nm) and macroporosity (15 μm), respectively. In a different approach, fragile scaffolds of bacterial cellulose aerogels were reinforced by incorporation of PLLA or cellulose acetate through antisolvent precipitation followed by sc-drying.[62]

3.4. Sintering. The ability of $scCO_2$ and compressed CO_2 to plasticize several polymers can be exploited to prepare scaffolds by sintering/consolidation of adjoining polymer particles and fibers.[63] In contrast to other state-of-the-art sintering methods (heat-sintering, solvent vapor treatment, solvent/nonsolvent sintering method, nonsolvent sintering technique),[63a,64] supercritical and compressed CO_2 sintering avoids the use of high temperatures and organic solvents. The extent of sintering is a function of the CO_2 sorption into the biopolymer, which is mainly influenced by the operating pressure and temperature as well as the exposure time (Figure 9). CO_2 exposure time should be low enough to be able to swell and plasticize only the surface of the microspheres and avoid oversintering (i.e., plasticization of the entire polymeric matrix).

This technique was applied to prepare PLGA scaffolds for tooth implants that perform as an exact copy of the extracted tooth root for immediate wound closure just after tooth extraction.[63b,65] PLGA porous scaffolds were prepared in four steps: (i) spinning of PLGA in a extruder to get fibers of 70 μm diameter; (ii) relaxation of the PLGA fibers under CO_2 at 5.5 MPa during 15 s to promote fiber contraction, folding, and aggregation; (iii) milling and sieving of the fiber aggregates into 700−1400 μm particles; (iv) consolidation of the PLGA particles located in a tooth root replica mold under CO_2 at 5.5 MPa during 15 s. The obtained scaffolds had an elastic modulus of 38.1 ± 7.7 MPa and total porosity of 60−65% with a high interconnectivity (ca. 98% of open pores) and mean pore sizes of 100 μm.

PLGA (50:50) microspheres (140, 175, and 240 μm) have been used to obtain sintered scaffolds for cartilage tissue engineering (CO_2 at 298 K, 1.3−3.0 MPa for 4 to 60 min).[63a] The extent of the sintering and the mechanical properties (elastic modulus) inversely correlated to PLGA intrinsic viscosity and particle size. Culture of porcine chondrocytes on the scaffolds led to cartilage-like matrix formation in 3 weeks. Further optimization studies revealed that 2.5 MPa is the ideal operating pressure (for a 1 h-period of CO_2 soaking) in terms of mechanical integrity and porosity (38−41%).[66] Lower pressure values gave low mechanical resistance, whereas higher values resulted in oversintering. Compressive modulus of the sintered PLGA scaffold decreased when increasing the L/G ratio from 50:50 to 85:15. Human MSCs seeded on these scaffolds located both on the surface and in the inner part of the construct. In a different embodiment, sintered PLGA (50:50) scaffolds incorporating human MSCs were prepared following a similar procedure, showing good cell viability.[63a] The processing time in this case was only 4 min to minimize the sterilization effect of CO_2 at longer durations. Finally, the combination of CO_2 sintering plus water leaching of PLGA microspheres (ca. 200 μm) containing NaCl particles (200−250 μm; 50 wt %) increased the porosity of the resulting scaffold in 58.4% with respect to that without salt, but the stiffness of the scaffold diminished.[66]

PCL scaffolds with 45% of porosity were obtained by supercritical sintering (4.8 MPa, 318 K, 4 h) of PCL microspheres (200 μm).[67] The lower CO_2 sorption in PCL than in PLGA under subcritical conditions was compensated by raising pressure, temperature, and soaking time in order to get similar porosities to those of PLGA-sintered scaffolds. Alternatively, PCL spheres can be subjected to $scCO_2$ (at short soaking times) to increase the CO_2 sorption in the polymer and reduce the processing time (Figure 9). PCL scaffolds were seeded with human bone marrow MSCs for 6 weeks showing mechanical stability and structural integrity in the culture medium during this period of time.[67] ALP activity in

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osteogenic medium of PCL scaffolds showed an increase in 3.4 times from week 0 to week 6, although results on osteogenic differentiation were worse than those obtained for the control sample (PLGA 50:50).

4. OTHER SUPERCRITICAL TECHNIQUES FOR PREPARATION OF INTERMEDIATES FOR SCAFFOLDS

Supercritical fluid technology can also be used to implement alternative processing methods of intermediate components (building blocks), such as microparticles and individual fibers, that can act as (micro)carriers of bioactive compounds once incorporated to the scaffolds.[68]

4.1. Supercritical Emulsion Extraction (SEE). SEE allows the production of uniform particles of sizes ranging between 100 nm and a few microns by extraction of the organic solvent (typically ethyl acetate or dichloromethane for PLGA) of w1/o/w2 double emulsions using $scCO_2$ as antisolvent (Figure 10).

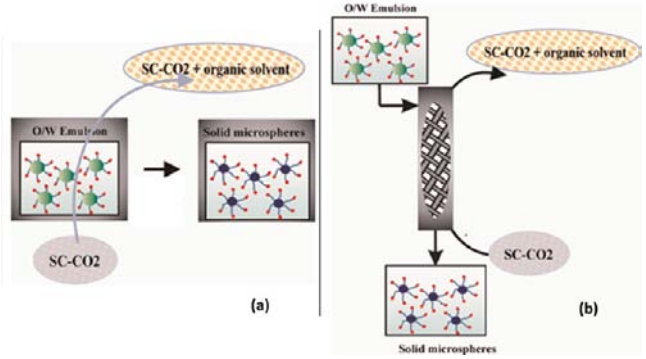

Figure 10. Sketch of a SEE process (a) in batch and (b) in continuous operating mode for the processing of aqueous dispersions of synthetic polymer microspheres. Adapted from ref 70a with permission of John Wiley and Sons.

The SEE process overcomes the drawbacks observed for other techniques (spray-drying, phase separation, solvent evaporation, or Prolease process among others[69]) when processing protein particles regarding temperature conditions, particle size control, process yield, solvent impurities, or scalability.[70] For example, batches of solvent-free PLGA microspheres containing lysozyme were prepared from emulsions controlling stirring rate (820 to 30 000 rpm) and polymer concentration (0.01 to 0.20 g PLGA per gram of solvent) and applying various lysozyme incorporation approaches (w1/o/w2 double emulsion, s/o/w suspension emulsion, and *in situ* s/o/w suspension emulsion).[70b] SEE conditions were kept constant during all experiments (313 K, 0.8 MPa, 80 g CO_2/min). Under these conditions, PLGA is in liquid or rubbery state and forms droplets that become solid microspheres after the solvent removal. High polymer concentration and low stirring rates led to greater particles with a broader distribution. *In situ* suspension emulsion provided the highest encapsulation efficiency (48.5%), compared to the double emulsion approach (10.9%).

SEE in continuous mode was applied to prepare PLGA microparticles loaded with growth factors (hVEGF and hBMP2) (Figure 10b).[71] The continuous layout improves material reproducibility and process yield with respect to the discontinuous setup.[70a] Briefly, a w1/o/w2 double emulsion containing PLGA in the oil (ethyl acetate) phase and GF solutions as w1 phase was fed (top) into a packed column in countercurrent with $scCO_2$ (8.0 MPa, 311 K) (bottom). Ethyl acetate was extracted with $scCO_2$ and the resulting water suspension containing 2 μm GF-loaded PLGA particles (encapsulation efficiency 80%) was collected at the bottom of the extraction column. PLGA microspheres containing hVEGF and/or hBMP2 were further dried by freeze−drying, and then incorporated into Ca^{2+}-alginate scaffolds containing human MSCs cells to evaluate the ability to modify cellular behavior, namely, osteoblastic differentiation. Results showed that mineralization and cell differentiation into osteoblasts were more significant when using dynamic culture rather than a static bioreactor. Osteocalcin mRNA expression was higher for scaffolds containing hBMP2 or combination of the two GFs, confirming the dominant effect of hBMP2 on MSCs osteoblastic differentiation.[72]

4.2. Particles from Gas Saturated Solutions (PGSS) Process. This method allows the processing of solvent-free particles by means of the plasticization of the polymer with $scCO_2$ under near ambient conditions (308 K) followed by a sudden depressurization through a restrictor.[23] Fillers and other bioactive admixtures (hydroxyapatite, enzymes, drugs)[73] can be homogeneously incorporated in the particles by mixing with the plasticized polymer prior to depressurization. The fine powder can be poured into a mold and cured to be used as a bone implant.[73e]

The PGSS technique is being used for preparation of PLGA and PLLA microparticles (50−100 μm) containing human growth hormones, which remain 100% active.[74] The formulation, intended for subcutaneous injection (once every 2 weeks to sustain serum hGH levels), has completed the preclinical trials in rats and monkeys.[74,75] Subsequent γ-irradiation for sterilization may alter hGH−polymeric matrix interactions, favoring the migration of hGH to the microparticle surface. PGSS has been also applied to prepare PCL particles (24−50 μm) containing fluorescein with mean particle size and particle size distribution tunable by the bioactive admixture/PCL ratio, the pre-expansion pressure and, to a lesser extent, the pre-expansion temperature.[23,76]

4.3. Particle and Fiber Formation Using Supercritical Antisolvent Processes. Particles and fibers of synthetic polymers (PLLA, PLGA, PMMA, PLLA/PLGA, PMMA/PCL) have been produced using different supercritical antisolvent techniques (SAS,[77] SEDS,[78] and PCA[51]). Briefly, synthetic polymer solutions (usually in dichloromethane) are injected through a nozzle into a chamber containing the nonsolvent ($scCO_2$), resulting in supersaturation and subsequent polymer precipitation. Several operating variables such as composition and concentration of polymers solution and liquid flow rate and nozzle design may have a significant impact on particle size distribution (mean size 2 to 20 μm) and on residual organic solvent content. Incorporation of bioactive compounds (paclitaxel and indomethacin) and inorganic fillers (Fe_3O_4 NPs) has been reported in the literature.[78,77b] Loading of GFs seems technically problematic due to the step of dissolution of the polymer in organic solvents.

Polymer fibers can be obtained using semidiluted and concentrated polymer solutions, which promote the entanglement of the polymer chains upon precipitation.[51,77c] Further postprocessing (e.g., thermal fiber bonding) may lead to 3D-scaffolds. Inorganic fillers (TiO_2, hydroxyapatite) can be optionally embedded in the fibers.[51] Loading of ketoprofen

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has been attempted, but the high affinity of $scCO_2$ for this anti-inflammatory drug resulted in low incorporation yields (<10%).

5. OTHER USES OF SUPERCRITICAL FLUIDS IN THE PROCESSING OF MATERIALS FOR REGENERATIVE MEDICINE

5.1. Supercritical Sterilization. Implementation of $scCO_2$-based sterilization methods may avoid alteration of the properties and structure of biomaterials that occur when treated with common sterilization methods (γ-irradiation, steam, e-beam, ethylene oxide, and hydrogen peroxide plasma). Sterilization efficacy of $scCO_2$ lies in lowering of cytoplasmic pH through carbonic acid formation, cell membrane damage, extraction, or inactivation of essential enzymes and shear forces upon depressurization, among others.[63a,79] Moreover, the sterilization time can be significantly reduced due to the high diffusivity of $scCO_2$. The effect of operating parameters (temperature, pressure, processing time) on sterilization efficacy has been evaluated in detail.[79a,80] Nevertheless, $scCO_2$ solely seems to be able to provide the sterility assurance level of 10^{-6} at sufficiently low temperatures and cycle times, at least when resistant bacterial spores are present. Addition of coadjuvants such as peracetic acid, trifluoroacetic acid, or hydrogen peroxide has been shown to improve the sterilization efficacy, with a subsequent shorter exposition of the material (e.g., bone) and thus better maintenance of its mechanical properties.[79b,80a,81]

5.2. Supercritical Cleaning. In general, $scCO_2$ treatment does not alter biochemical and biomechanical properties of bone fragments, tendons, or even acellular dermal matrices.[81a] Bone treatment usually involves defatting before transplantation in order to decrease adverse immunological responses, to improve biocompatibility and increase osteoconductivity. Efficacy of $scCO_2$ treatment for bone marrow removal is similar or even superior to that of conventional methods using toxic organic solvents, as confirmed in clinical trials of xenogenic bone implants in pigs.[82] Additives or cosolvents are not needed, which prevents the presence of residual solvents in the processed bone. Human bone graft stiffness is not influenced by the supercritical treatment, but bone graft strength and energy dissipation are significantly reduced with respect to the fresh bone.[83] A post-treatment with H_2O_2 for bone deproteination can be carried out in the case of allogenic implants to decrease immunogenicity.[84] Clinical trials in pigs with ovine bone implants showed excellent osteointegration of the treated bone fragment after eight months. Finally, a one-pot supercritical defatting-plus-sterilization of micronized human bone has been proposed with good results, although the risk of immunogenicity still has to be addressed.[85]

6. CONCLUSIONS AND PERSPECTIVES

Supercritical fluid technology has already become a realistic alternative to manufacture scaffolds with tailor-made morphology, able to overcome the usual drawbacks (use of organic solvents, vigorous mechanical agitation, high temperature and poor control of the 3D-structure) arising with conventional techniques. Gentle operating conditions, compatible with the incorporation of bioactive compounds and cells, along with the low cost of $scCO_2$ and the absence of downstream processes revert in a technology for regenerative medicine with high benefit-to-cost ratio.

Incorporation of inorganic admixtures and GFs in scaffolds using supercritical fluids helps to enhance the mechanical properties as well as cell proliferation and differentiation. However, the interface of the polymeric matrix with the admixtures is not always well designed leading to poor embedding in the matrix and the formation of heterogeneous materials with agglomerates, and further optimization is still needed. Although some attempts of tuning the polymeric matrix−filler interface have already been reported in the literature,[86] the biofunctionalization and surface modification of either the biopolymer or the admixtures with new chemical functionalities by f.i. supercritical impregnation/grafting should be further explored.

Finally, the incorporation of novel ingredients in the scaffolds to render new therapeutic effects and improved performance to the construct has to be studied. In this sense, a one-step process of scaffold formation and incorporation of cells using $scCO_2$ has already been reported.[87] This processing approach needing further development will avoid a supplementary cell seeding step and would likely accelerate the cell in-growth process in the early proliferation stages. In addition to GFs, the incorporation of small drugs can provide novel performances to the implant, f.i. regarding prophylaxis to mitigate foreign body reactions and to prevent biofilm formation, whereas the scaffold can provide site-specific, controlled release.[88] Several model systems incorporating drugs in scaffolds using supercritical fluids, during scaffold formation or by postprocessing, have shown interesting release profiles and encapsulation yields. The synergistic effect of these drug-containing scaffolds seems very promising but still needs further intensive studies regarding each particular case of application before reaching the clinical arena.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ALP, alkaline phosphatase; ASES, aerosol solvent extraction system; BSA, bovine serum albumin; ECM, extracellular matrix; GAS, gas antisolvent; GDP, gross domestic product; GF, growth factor; GRAS, generally recognized as safe; hBMP, human bone morphogenetic proteins; hBMSC, human bone marrow stromal cells; hMVEC, human dermal microvascular endothelial cells; hVEGF, human vascular endothelial growth factor; MSCs, mesenchymal stem cells; NPs, nanoparticles; P_c, critical pressure; PCA, precipitation with compressed antisolvent; PCL, poly(ε-caprolactone); PGSS, particles from gas saturated solutions; $P_{D,L}LA$, poly(D,L-lactic acid); PLA, poly(lactic acid); PLGA, poly(lactic-*co*-glycolide); PLLA, poly(L-lactic acid); RESS, rapid expansion of a supercritical solution;

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rt, room temperature; SAS, supercritical antisolvent; SBF, simulated body fluid; $scCO_2$, supercritical carbon dioxide; sc-drying, supercritical drying; SEDS, solution enhanced dispersion by supercritical fluids; SEE, supercritical emulsion extraction; T_c, critical temperature; TCP, tricalcium phosphate; T_g, glass transition temperature; t_R, residence time; 3D, three-dimensional

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