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The Future of Carbon Dioxide for Polymer Processing in Tissue Engineering

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The use of CO_2 for scaffold fabrication in tissue engineering was popularized in the mid-1990s as a tool for producing polymeric foam scaffolds, but had fallen out of favor to some extent, in part due to challenges with pore interconnectivity. Pore interconnectivity issues have since been resolved by numerous dedicated studies that have collectively outlined how to control the appropriate parameters to achieve a pore structure desirable for tissue regeneration. In addition to CO_2 foaming, several groups have leveraged CO_2 as a swelling agent to impregnate scaffolds with drugs and other bioactive additives, and for encapsulation of plasmids within scaffolds for gene delivery. Moreover, in contrast to CO_2 foaming, which typically relies on supercritical CO_2 at very high pressures, CO_2 at much lower pressures has also been used to sinter polymeric microspheres together in the presence of cells to create cell-seeded scaffolds in a single step. CO_2 has a number of advantages for polymer processing in tissue engineering, including its ease of use, low cost, and the opportunity to circumvent the use of organic solvents. Building on these advantages, and especially now with the tremendous precedent that has paved the way in defining operating parameters, and making the technology accessible for new groups to adapt, we invite and encourage our colleagues in the field to leverage CO_2 as a new tool to enhance their own respective unique capabilities.

Introduction

Carbon dioxide has found enormous uses in virtually all fields of science and research over the past several decades. Its use as a supercritical fluid, along with its plasticizing and solvent properties, has enabled it to be used in a wide variety of tissue engineering and regenerative medicine applications.[1–6] In the field of tissue engineering, the majority of current processing techniques for scaffold fabrication use organic solvents and/or high temperatures.[7] CO_2 technology provides an alternative to these methods with many applications described in the literature.[8] It is also interesting to note that under specific conditions, CO_2 has also been used for sterilization.[9–14]

Colton and Suh[15] in 1987 reported one of the first uses of CO_2 and N_2 to produce foams of polystyrene. The first mention of CO_2 foams for tissue engineering scaffolds can be found in a 1991 patent,[16] a technique that was first brought to the tissue engineering literature by Mooney *et al.*[17] in 1996, who made porous disks of poly(D,L-lactic-co-glycolic acid) by exposure to CO_2 for prolonged periods of time. While porosities up to 93% were obtained, there was only partial interconnectivity between the pores. They also observed the presence of a nonporous skin layer, which had also been observed earlier by others,[18,19] and which also turned out to be an important challenge to overcome for other groups that followed.

The use of supercritical CO_2 for generating porous polymeric foams has generated significant interest over the years. Several advancements have been made in the tissue-engineering field since its first use by Mooney *et al.*[17] Most of the techniques utilizing supercritical fluid technology in pharmaceutical and drug delivery applications have been reviewed eloquently and thoroughly by the team of Howdle and coworkers[4–6] from the United Kingdom. In addition, a very recent review by Reverchon and Cardea[3] from Italy covered an impressive variety of techniques using CO_2 in scaffold fabrication for tissue engineering, including foaming (with or without particulate leaching), supercritical assisted phase separation (e.g., thermal induced phase separation), solvent elimination (e.g., drying ionic liquid–polymer mixtures), supercritical fluid-assisted electrospinning, and replacing organic solvents with supercritical CO_2 in polymerization of high internal phase emulsions. Hence, this review will focus on recent (past $\sim$5 years) developments in tissue engineering applications, and also mention the use of subcritical CO_2 for polymer sintering in scaffold fabrication, emphasizing that not all CO_2 applications must be supercritical.

The impetus for this review is that with the rapid growth in the number of advanced biomaterials and fabrication

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methods for scaffolds in tissue engineering, there are a number of advantages and opportunities with CO_2 of which many investigators in the tissue engineering community are not aware. CO_2 processing is relatively straightforward and affordable to incorporate in a laboratory, and we encourage both industry and academia to take another look at what CO_2 may add to their particular application.

Properties of CO_2

A supercritical fluid is a dense-phase fluid whose pressure and temperature are above its critical point. At the critical point of a substance, a single phase occurs that has a liquid-like density and a gas-like viscosity and compressibility.[20] It is important to note that above the critical temperature, compression yields a continuous increase in fluid density without condensation to a liquid state. These properties can be easily tuned by changes in both pressure and temperature, as opposed to conventional organic solvents whose properties are much less dependent on temperature and almost unchanged with pressure. Supercritical fluid and dense-phase gas (near-critical) technology is an area of intense fundamental and applied research, especially as an environmentally benign solvent alternative. CO_2 is the most often used substance, in part because it has a relatively low critical temperature and pressure (T_c=31.1°C and P_c=73.8 bar), which makes it suitable for processing thermosensitive compounds. Furthermore, it has the additional advantages of being inexpensive, nontoxic, and nonflammable. The recovery of final products and removal of CO_2 can be done easily with no residue left behind.[21]

CO_2 helps in reducing the polymer melt viscosity[22–26] by decreasing the glass transition temperature (T_g) or melting temperature (T_m) due to its high solubility in polymers.[27] The molecular structure and morphology of polymers greatly influence CO_2 solubility and diffusivity. The carbonyl or ether groups in the backbone or on side chains of a polymer interact with CO_2 and help with the dissolution of CO_2 within the polymer.[28–30] Polymers that have ether groups in their backbone structure such as poly(ethylene glycol) (PEG) have been shown to have stronger interactions with CO_2 than polyesters, which have ester functional groups in their main chains. This has been attributed to weak Lewis acid–base interactions between them.[31] Steric hindrance can also influence solubility of CO_2 in the polymer. In the case of poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), both polymers have a similar chemical structure in their main chains, but the solubility of CO_2 is higher in PLA than in PLGA, as the accessible free volume caused by methyl pendant groups improves solubility. Therefore, with increasing glycolic acid content in PLGA copolymers, the solubility of CO_2 decreases.[32] A pair of studies from the same group more closely examined this effect, comparing PLGA at lactic acid:glycolic acid ratios of 85:15, 75:25, 65:35, and 50:50 with pure PLA.[30,33] In comparing similar molecular weights of 50:50 PLGA with pure PLA, CO_2 sorption was 20%–30% higher in PLA at subcritical pressures and 45%–55% higher at supercritical pressures,[33] while different molecular weights of PLA had a negligible effect on CO_2 sorption.[30,33] In agreement with these CO_2 sorption observations, they also found that PLA experienced a greater viscosity reduction than PLGA.[30] Very recently, this group also identified a novel window of high miscibility of PLA/PEG blends in supercritical CO_2 when the PEG weight content was about 8%–25%.[34]

For highly crystalline polymers such as PLA (98:2, 20% crystallinity), poly(glycolic acid) (PGA), PEG, and polycaprolactone (PCL), CO_2 has a relatively low solubility and slower diffusivity at temperatures below their T_g or T_m. With amorphous poly(D-L-lactic acid) ($P_{DL}LA$) and PLGA, larger free volumes compared to PGA allow more CO_2 to dissolve and reduce the T_g and T_m.[35] Collectively, all of the aforementioned properties in this section have widely enabled CO_2 to be used in the field of tissue engineering for various applications such as in the production of polymer scaffolds and composites, encapsulation and release of bioactive compounds, and encapsulation of mammalian cells.[4]

Preparation of Three-Dimensional Scaffolds in Tissue Engineering

Porous three-dimensional (3D) scaffolds used in tissue engineering ideally help with cell attachment, differentiation, and proliferation to regenerate a given tissue of interest.[36–38] Particularly for musculoskeletal tissues, biodegradable constructs must have adequate mechanical integrity to support the load-bearing activities of the tissue. To date, several methods have been reported in the literature for the preparation of 3D scaffolds, some of which include processes such as solvent casting with particulate leaching,[39] compression molding,[40] freeze drying,[41] heat sintering,[42,43] injection molding,[44] layer-by-layer printing[45] or sintering,[46] and electrospinning.[47] Each of these methods has its own advantages, but these methods typically make use of large amounts of organic solvents and/or exposure to elevated temperatures. Supercritical and dense-phase fluid technologies provide an attractive alternative to these traditional methods of scaffold fabrication. In this section, we discuss the strategies that have been applied for creating 3D porous polymer-based scaffolds and their applications from a tissue-engineering perspective. Among several scaffold design parameters, select few such as pore size, pore interconnectivity, porosity, and processing conditions are of high interest.[2,33,48,49] The pore network is of paramount importance, because it will govern cell infiltration and nutrient/waste transport, but must not come at the expense of mechanical integrity for musculoskeletal tissues, where mechanical performance is also of crucial importance.

Gas Foaming

Gas foaming is one of the most commonly used techniques making use of supercritical fluid technology for the fabrication of 3D scaffolds for tissue engineering. It was first described by De Ponti *et al.*[16] in a 1991 patent where gas foaming was used for making scaffolds with closed pore structures from biodegradable poly(α-hydroxyacids) such as P_LLA, $P_{DL}LA$, PGA, and PLGA.[16] In this technique, the polymer is saturated with CO_2, which at high pressures causes it to plasticize by reducing the glass transition temperature. This reduction in T_g of the polymer is achieved as a result of the intermolecular interactions between CO_2 and the polymer. Greater T_g depression is observed in polymers that have stronger interactions. After saturation of the polymer with CO_2, rapid depressurization causes thermodynamic

instability and results in the formation of nucleated gas cells that give rise to pores within the scaffold. This technique is mainly applicable for amorphous and semicrystalline polymers that have a higher affinity for CO_2 when compared to crystalline polymers along with a relatively low T_g or T_m.[21]

Goel and Beckman[18,19,50–54] contributed a series of publications in the early-to-mid 1990s on fabricating polymeric foams with supercritical CO_2. Noteworthy in these publications was the focus given to the nucleation process. By assuming a homogeneous liquid state of the CO_2–polymer system (justified by the high pressures employed), they deemed the application of classical nucleation theory to be appropriate.[18] Growth of these nuclei was modeled based on mass and momentum transfer equations for gas–polymer systems. Briefly, they were able to link their experimental data to nucleation theory in adjusting pressure, temperature, and saturation time to control the final foam structure (including the nonporous skin layer). It is worth noting that they observed some deviation from behavior predicted by the classical nucleation theory at the lower range of investigated pressures (103 bar), which they attributed to possibly heterogeneous nucleation. Their work has provided an important set of equations to predict equilibrium pore size and pore density as functions of temperature and CO_2 pressure.[50]

Mooney *et al.*[17] popularized the supercritical CO_2 foaming process by making porous poly(D,L-lactic-co-glycolic acid) disks by exposure to CO_2 for 3 days, followed by rapid depressurization, which resulted in gas nucleation and formation of pores up to 97%. However, this process also formed a nonporous skin layer over the entire outer surface of the polymer matrix, which is not suitable for cell adhesion.[17] To overcome this issue, Mooney and his coworkers[55] introduced salt (NaCl) particles to the polymer solution before gas foaming. Leaching of this porogen after fabrication of the polymer foam created an interconnected open pore network. The degree of porosity and interconnectivity was regulated by altering the salt/polymer ratio and the salt particle size. They found that the polymer disks containing a large percentage (95%) of large NaCl particles did not have an external, nonporous skin over the scaffold surface.[55] Several improvements to the conventional gas foaming technique have been made since then. For example, Barry *et al.*[56] in 2004 simply removed the skin layer from the scaffolds before cell culture when they used the gas-foaming method to create poly(ethyl methacrylate)/tetrahydrofurfuryl methacrylate foams that were found to have about 87% porosity with nearly 57% open pores. An additional improvement in this study over the aforementioned pioneering study by Mooney *et al.*[17] was that Barry *et al.*[56] were able to achieve pore interconnectivity without particulate leaching. These foams supported bovine chondrocyte proliferation by displaying increased glycosaminoglycan synthesis and retention of rounded cell morphology.

Nevertheless, the use of particulate incorporation has continued. For example, Salerno *et al.*[57] combined gas foaming with microparticulate templating to achieve open-pore biodegradable foams made of PCL with a controlled porous architecture. Composites of PCL were combined with micrometric NaCl particles in concentrations ranging from 70/30 to 20/80 wt% at 70°C for 3 h at a pressure of 65 bar. It was observed that porosity, pore size, and pore interconnectivity were controlled by optimizing the processing parameters. Spatial gradients of pore size and porosity were achieved within the same scaffold by using a microparticle concentration gradient of NaCl. In addition, addition of microparticulate silica has been attempted to improve the pore interconnectivity in scaffolds prepared by supercritical CO_2 foaming.[1] It was found that by increasing the amount of silica particles in the polymer, smaller pores could be obtained that had greater interconnectivity. Porosity was not affected by the presence of silica during CO_2 foaming.

Gualandi *et al.*[58] prepared polymeric foams of ω-pentadecalactone and ε-caprolactone using supercritical CO_2 foaming. They observed that foaming was possible at a temperature greater than the melting temperature of the copolymer. The pore diameter and porosity were found to be dependent on the cooling rate. A cooling rate of 0.23°C/min resulted in a pore diameter of 225 μm with 70% porosity. Control over pore size and interconnectivity was achieved by altering the rate of depressurization. Further details about the processing conditions have been listed in Table 1.

Mathieu *et al.*[59] applied supercritical CO_2 gas foaming technology to produce composite cellular structures having a heterogeneous architecture of pores in PLA foams also containing hydroxyapatite (HA). They observed that addition of HA resulted in more heterogeneous foams than with β-tricalcium phosphate (β-TCP). Ceramic particles of HA and β-TCP were distributed in the pore walls of the composite foams, thereby providing an efficient reinforcement of the matrix. These foams had an average pore size from 200 to 400 μm with porosities between 78% and 92%. Tsivintzelis *et al.*[60] found that crystalline polymers such as PCL can undergo supercritical CO_2 foaming with the addition of small amounts of organic solvents such as ethanol. Addition of ethanol resulted in more uniform cell structures in the scaffolds than those prepared using CO_2 alone, and also resulted in larger pore formation. However, all of the samples had a dense unfoamed skin usually apparent with the gas foaming technique.

Reverchon *et al.*[61] prepared a foamed poly(L-lactic acid) (PLLA) scaffold that had an elevated porosity of above 90% with pore interconnectivity. Regarding mechanical integrity, the compressive modulus (Young's modulus determined from tensile experiments) was 81 kPa. The scaffolds were prepared by a three-step process where a polymeric gel loaded with a solid porogen (in this case fructose) was first formed. The next step involved drying of the gel with supercritical CO_2, followed by washing with water to eliminate the porogen. Pore size was controlled by the size of the porogen added during the process.

Tai *et al.*[49] identified the trends in pore growth and porosity by analyzing the effect of various parameters such as soaking time, soaking pressure, soaking temperature, depressurization rate, molecular weight, and chemical composition of the polymer. They prepared a porous structure of PLA and PLGA by a CO_2 foaming process. The scaffolds had the presence of a nonporous skin layer. The results demonstrated that pore size decreased with increasing glycolic acid content for PLGA scaffolds. However, increasing glycolic acid content also resulted in lower porosity, with a maximum of 78% porosity obtained from PLGA scaffolds. Constructs made of polymers with low molecular weight (i.e., PLGA 85:15 with 15 kDa and PLGA 75:25 with 13 kDa) were found to be very fragile. A higher pressure and a longer

Table 1. Processing Conditions for Scaffold Fabrication with CO_2

References	Scaffold material	CO_2 process conditions	Scaffold fabrication method	Major results	Applications
White *et al.*[2]	Poly (D,L-lactic acid) ($P_{DL}LA$) (52, 25 and 15 kDa)	Saturation pressure: 232 bar Saturation temperature: 35°C dP/dt = 23.2, 7.7, 5.2 and 3.9 bar/min	Gas foaming	1. Wider pore-size distribution with larger, interconnected pores was observed at lower depressurization rates of 5.2 and 3.9 bar/min. Pore size was also found to decrease with increasing molecular weight. 2. Scaffolds fabricated at the faster depressurization rate (23.2 bar/min) were seen to have a lower pore interconnectivity, while the slower depressurization rates (5.2 and 3.9 bar/min) allowed more time for the pores to grow and coalesce, resulting in a higher pore connectivity. 3. Compressive testing showed that high-MW $P_{DL}LA$ (52 kDa) had elastomeric properties (a linear elastic region, a collapse plateau region, and a densification region), while the lower-MW $P_{DL}LA$ (25 and 15 kDa) was brittle.	$P_{DL}LA$ (57 kDa) has potential for bone tissue engineering applications.
Salerno *et al.*[57]	PCL with micrometric NaCl particles	Soaking pressure: 65 bar Soaking temp.: 70°C Soaking time: 3 h	Gas foaming and microparticulate templating	1. Microparticle concentration gradients of NaCl were used to prepare open-pore foams with spatial gradients of porosity and pore size. 2.Varying the microparticle concentration resulted in the control of porosity (in the range 78%–93%) and pore size from 90 to 10 μm.	This technique can be tailored to design the microarchitecture of open-pore foams with respect to desired applications.
Gualandi *et al.*[58]	Copolymer of ω-pentadecalactone (PDL) and ε-caprolactone (CL), poly (PDL-CL)	Soaking pressure: 230 bar Soaking temp.: 90°C Soaking time: 20 min dP/dt = 4 bar/min dT/dt = 0, 0.15, 0.23 and 0.27°C/min	$scCO_2$ gas foaming	1. Cooling rate of 0.23°C/min showed best results with pore diameter of 255 μm and 70% porosity. 2. Short soaking time (1 min) led to a nonporous core with a porous shell; t_{soak} of 10 min led to pore size of 155 μm and 57% porosity. Increasing t_{soak} to 20 min decreased pore size to 110 μm. 3. Better-average pore size and interconnectivity were obtained by decreasing the rate of depressurization. 4. The compressive modulus values obtained were in the same range as that of articular cartilage.	(PDL-CL) can find applications in cartilage tissue engineering.
Mathieu *et al.*[59]	PLA with hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP)	Soaking pressure: 100–250 bar Soaking temp.: 195°C Soaking time: 10 min dP/dt = 2–15 bar/s dT/dt = 3–7°C/s	$scCO_2$ gas foaming	1. Composite cellular structures obtained had a heterogeneous architecture with more closed pores. 2. A 5% filler content is the maximum possible to obtain foams with adequate microstructure (especially pore size and interconnections). 3. Addition of HA resulted in more heterogeneous foams than with β-TCP. Ceramic particles got distributed in the pore walls of the composite foams, thereby providing an efficient reinforcement of the matrix. 4. Filled foams had modulus of up to 250 MPa with average pore size from 200 to 400 μm and porosities between 78% and 92%.	Applications in bone tissue engineering.

(*continued*)

Table 1. (Continued)

References	Scaffold material	CO_2 process conditions	Scaffold fabrication method	Major results	Applications
Tsivintzelis *et al.*[60]	PCL	Soaking pressure: 123–205 bar Soaking temp: 35–45°C	$scCO_2$ gas foaming with ethanol as blowing agent	1. Crystalline polymers can undergo $scCO_2$ foaming by addition of small amounts of organic solvents. 2. Pure CO_2 as blowing agent resulted in a heterogeneous structure with regions of different pore sizes and densities. In contrast, constructs prepared using CO_2 and ethanol exhibited more uniform cell structures. This also resulted in larger pore formation. 3. All samples had dense unfoamed skin usually seen with the gas foaming technique.	—
Tsivintzelis *et al.*[63]	PLLA	Saturation pressure: 100, 165, 230 bar Saturation temp.: 45°C Saturation time: 2.5 h dP/dt: 1 bar/min Solvent: Dichloromethane	Phase inversion using $scCO_2$ antisolvent	1. Pore size decreased with pressure variation from 100–230 bar. Decrease in temperature (or increase in pressure) increases density and solvent power of CO_2. 2. Lesser initial polymer concentration leads to larger pore formation.	—
Reverchon *et al.*[64]	PMMA	Saturation pressure: 200 bar Saturation temp.: 45°C Saturation time: 45 min Solvent: DMSO and acetone	Phase inversion using $scCO_2$ antisolvent	1. Increase in polymer concentration leads to decrease in pore size in both solvents. 2. L-L demixing is the controlling mechanism. 3. Pore interconnectivity observed in acetone but closed pores in DMSO. 4. Increase in pressure from 150 to 250 bar causes decrease in pore diameter from 15 to 7 μm. Increase in temperature from 35°C–65°C increases pore size from 8 to 12 μm.	PMMA loaded with an antibiotic, amoxicillin, for drug delivery applications.
Duarte *et al.*[65]	Starch and poly (L-lactic acid) (SPLA)	Saturation pressure: 200 bar Saturation temp: 55°C Saturation time: 45 min dP/dt: 5 g/min	Phase inversion using $scCO_2$ antisolvent	1. Polymer matrices are bicontinuous surfaces that have micro- and macropores; highly interconnected pores. 2. Porosity up to 66% and pore size of macropores is 200 μm, micropores is 20–50 μm. 3. Up to 90% swelling and weight loss of about 25% observed after 21 days in solution.	SPLA loaded with dexamethasone to be used in bone tissue engineering applications.
Duarte *et al.*[66]	Chitosan	Saturation pressure: 150 bar Saturation temp.: 60°C Saturation time: 45 min dP/dt: 5 g/min	Phase inversion using $scCO_2$ antisolvent	Formic acid as a solvent resulted in 29% porosity and average pore size 62 μm; acetic acid: 47% porosity and 110-μm pore size; HFIP: 90% porosity and 600-μm pore size.	Drug delivery applications; tissue engineering of bone and cartilage.

MW, molecular weight; PCL; $scCO_2$ = supercritical CO_2; PLA, poly(lactic acid); PLLA, poly(L-lactic acid); PMMA, poly(methyl methacrylate); DMSO, dimethyl sulfoxide.

soaking time facilitated the production of smaller pores, as more CO_2 molecules diffused through the polymer matrix due to higher solubility, thus leading to a higher nucleation density. Larger pores were obtained by increasing the temperature, as this increased the rate of diffusion, which allowed pore growth. Slower rates of depressurization provided more time for pore growth and thus resulted in the formation of larger pores, whereas increasing the rate of depressurization typically resulted in a nonuniform pore structure. One may conclude that slower rates of depressurization (on the order of an hour rather than on the order of minutes) may be more desirable for creating scaffolds for tissue engineering.

Baker *et al.*[62] prepared porous resorbable polymer constructs by means of supercritical CO_2 processing that had structural and mechanical properties similar to human bone. A porous poly-D-lactide-co-glycolide construct was soaked in supercritical CO_2, followed by rapid depressurization. The constructs were then freeze-fractured with liquid nitrogen in the vertical and perpendicular directions. A lower CO_2 processing temperature of 35°C helped form larger pores with thicker pore walls, while processing at 100°C formed relatively smaller pores with a very low extent of pore interconnectivity. Using different CO_2 processing pressures had a similar effect on the pore architecture. It was reported that all of the constructs had a dense cortical shell about 15–20 μm thick with an interconnected porous core with pore diameters in the range of 236–239 μm. Mechanical integrity and water uptake capacity were found to be dependent on the glycolic acid content of the polymer.

Gas foaming can produce open-cell, interconnected pores in a solvent-free process under the right conditions. However, the greater degree of porosity can have an effect on the mechanical integrity of the construct. White *et al.*[2] addressed this issue of optimizing porosity and mechanical strength. They formed foams made of different molecular weights of $P_{DL}LA$ (57, 25, and 15 kDa) and varied the depressurization rate. During depressurization, supersaturation of CO_2 occurred within the polymer, which led to nucleated bubble formation. It was observed that the rapid depressurization rate produced scaffolds with homogeneous pore distributions with closed pores. A decrease in depressurization rates resulted in wider pore distributions in the scaffolds with larger, interconnected pores. Compressive testing of these constructs showed that the higher-molecular-weight (MW) $P_{DL}LA$ (52 kDa) showed elastomeric properties (a linear elastic region, a collapse plateau region, and a densification region), while the lower-MW $P_{DL}LA$ (25 and 15 kDa) was more brittle in nature. The 52-kDa $P_{DL}LA$ showed potential for bone tissue engineering applications. Further details about improvements in processing conditions have been described briefly in Table 1.

Gas foaming is one of the most commonly used techniques for making scaffolds using CO_2. With gas foaming, a variety of conditions and parameters have been investigated with different materials, and common concerns being pore interconnectivity and presence of a skin layer. The concern of pore interconnectivity has been mitigated with the evolution of the technology, as processing conditions (pressure, venting time, soaking time, etc.) more conducive to tissue engineering have been employed. Regarding the skin layer, the rapid diffusion of the dissolved fluid out of the sample edges results in the formation of this dense, nonporous skin layer, which can be decreased by increasing the pressure.[51] The presence of this layer is not desirable for tissue engineering applications, and the most straightforward solution thus far has been to remove the skin manually, although the approach of using particulate matter to be leached out and effectively leave particulate-generated (as opposed to CO_2-generated) pores has been used. Perhaps the use of a mold with a nanotextured or nanoporous surface could be employed that would allow for CO_2 gas nucleation and/or escape at the periphery and eliminate the skin layer altogether. However, currently, the most desirable option is manual removal of the skin layer, which has a strong precedent in the literature to follow.

Phase Inversion: CO_2 as an Antisolvent

In the phase-inversion method, a polymer solution is cast onto an inert support that is then immersed into a bath containing nonsolvent for the polymer. Contact between the solvent and nonsolvent results in a phase separation. CO_2 is the most commonly used supercritical fluid that is being used as a nonsolvent. Using CO_2 also avoids a drying step in the end, thereby resulting in a dry product with minimal residual solvents. By tuning the process conditions such as pressure and temperature, the final structure of the product can be modified as needed.[8,21] The phase-inversion method using CO_2 as a nonsolvent has been used successfully for the preparation of different polymeric scaffolds (Table 1).

Tsivintzelis *et al.*[63] used the phase-inversion method to prepare PLLA foams. They observed that pore size decreased with pressure variation from 100 to 230 bar. Lesser initial polymer concentration led to the formation of larger pores. Reverchon *et al.*[64] also observed that pore diameter decreased (from 15 to 7 μm) with increasing pressure (from 150 to 250 bar) for poly(methyl methacrylate) foams. On the other hand, pore size increased from 8 to 12 μm on increasing the temperature from 35 to 65°C.

Duarte *et al.*[65] formed polymer matrices from starch and poly(L-lactic acid) by the phase-inversion method. The resultant scaffolds had a porosity of 66% with macropores of 200 μm in diameter and micropores of 20–50 μm in diameter. These constructs had a 90% swelling value and a weight loss of 25% after 21 days in culture. They later applied this method to form chitosan foams[66] with 29% porosity and an average pore size of 62 μm. Chitosan foams were found to be suitable for tissue engineering of bone and cartilage due to their physicochemical compatibility and biocompatibility.

Using a supercritical fluid as a nonsolvent during phase inversion helps in obtaining scaffolds that do not have any residual organic solvents. This approach has been used to form scaffolds from different polymeric materials and has found several applications.

Supercritical Fluid Emulsion Templating

With the method of supercritical fluid emulsion templating, concentrated oil-in-water emulsions can be phase separated to create porous scaffolds. A variety of porous hydrophilic scaffolds can be prepared using this technique. The final porous product can be recovered by removing the internal phase, which is the emulsion. This technique has been extended to supercritical CO_2-in-water emulsions as well. Butler *et al.*[67] used this method to stabilize the

CO_2-in-water (C/W) emulsions of acrylamide polymers by using perfluoropolyether surfactants and poly(vinyl alcohol). After polymerization, venting of CO_2 resulted in the formation of interconnected pores within the polymer scaffold. They found that increasing the volume fraction of the CO_2 internal phase increased porosity. It was also observed that by increasing the concentration of the surfactant, greater interconnectivity within the open pores could be achieved.[67] This method has been scarcely studied, and there is potential for others to use this method if they want to obtain porous hydrophilic scaffolds.

Electrospinning

Electrospinning is an intriguing method that has been used for the production of polymeric fibers from biomaterials and composites.[68,69] Here, an electric field is utilized to eject a charged polymer stream from a needle, which then results in the formation of microscale fibers under the influence of tangential stresses and bending instabilities.[68,70] The diameter of the viscoelastic jet can be reduced to produce micron- and nano-sized fibers by using the electrostatic repulsions between the surface charges.[71] Electrospinning has been used for a variety of applications in tissue engineering,[70] some of which include using electrospun scaffolds for cartilage replacement,[72–74] bone grafts,[75,76] and cardiac grafts.[77] Electrospun fibers can also be used for seeding stem cells,[78,79] and endothelial cells[80] to form a 3D cellular network.

Supercritical CO_2 can be used as a swelling agent for polymers and can help impregnate the scaffolds with desirable additives such as drugs and bioactive compounds. Ayodeji *et al.*[70] embedded electrospun PCL with carboxytetramethylrhodamine using near-critical CO_2 at a pressure of 34.4 bar for a period of 2.5 h. They found that the individual fibers remained intact and showed a distinct nonwoven fibrous network at a low temperature of 10°C, but at a higher temperature of 40°C, the microstructure of the fibers began to change. They also observed a significant distribution of carboxytetramethylrhodamine throughout the surface of the PCL. Encapsulation of a bioactive molecule using supercritical CO_2 helps to protect conformationally sensitive molecules from the shear forces present during the electrospinning process.[70]

Levit and Tepper[68] used supercritical CO_2 to produce PLA-electrospun fibers by using only electrostatic forces without the use of a liquid solvent. They found this new supercritical fluid-assisted electrospinning (SAES) technique useful for producing large- and small-diameter fibers.

In 2010, Liu *et al.*[69] combined the traditional electrospinning process with a precipitation with a compressed fluid antisolvent (PCA) method to produce micron- and submicron-sized polymeric fibers that had either a hollow or open-cell morphology. Supercritical CO_2 was used as the compressed fluid. Using this technique, they found that it was possible to obtain different fiber morphologies by simply adjusting the CO_2 pressure, and that high temperature and pressures in excess of 100 bar were not needed. They also suggested using this technique to encapsulate live cells to produce celloidosome fibers, citing the mild temperatures and pressures as supporting points.[69]

CO_2 offers electrospinning the advantage of obtaining different diameter fibers with open pore structures without using a liquid solvent. It also helps in encapsulating live cells and heat-sensitive compounds within the electrospun fibers.

Hydrogel Foaming Using CO_2

Hydrogels are highly hydrated polymeric materials that consist of hydrophilic polymer chains. The crosslinks between the polymer chains formed by various chemical bonds and physical interactions contribute to the structural integrity of the hydrogels.[81] Several studies have been reported in the literature that use high-pressure CO_2 for the foaming of polymers to form hydrogels.

Tsioptsias and Panayiotou[82] investigated the extent and mechanism of supercritical CO_2 sorption by chitin hydrogels and the production of pores within these hydrogels. Chitin gels were prepared by dissolving chitin in adimethylacetamide and LiCl mixture followed by extensive washing in distilled water. Crosslinking within the gel was achieved by exposure to glutaraldehyde vapor at room temperature. They found that CO_2 sorption by the gel was due to its dissolution in the water of the hydrogel. Foaming of the hydrogel was observed during the depressurization, but it immediately shrunk on exposure to air. They found that freeze-drying the sample immediately after depressurization helped to retain the initial porous structure formed during the foaming process. However, a dense outer skin was present on the surface of the porous hydrogels.[82]

In 2010, Tsioptsias *et al.*[83] proposed a mechanism for this hydrogel foaming technique. On depressurization, they proposed that there was heterogeneous nucleation at the polymer–water interface as well as homogeneous nucleation in the water phase, leading to the growth of pores. After depressurization, temporary stabilization was achieved by cooling. Freeze-drying led to complete stabilization of the structure. In comparison, during polymer foaming, there was only homogeneous nucleation in the polymer phase, which caused pore growth. Stabilization of the produced structure was achieved by vitrification.[83]

Annabi *et al.*[84] investigated the effect of supercritical CO_2 foaming on elastin-based hydrogels. Increasing the CO_2 pressure from 30 to 150 bar caused about a 60% increase in the hydrogel foaming ratio. It also accelerated the crosslinking time and facilitated coacervation, leading to enormous changes in the macro- and microstructures of the pores formed within the sample. Increasing pressure was also found to reduce the wall thickness and size of the pores. It induced channels within the structure of the elastin hydrogels that promoted fibroblast penetration and proliferation.[84]

CO_2 has been mainly used in hydrogels for the formation of pores within the scaffolds and for hydrogel foaming. Using CO_2 provides control over the microstructure and size of the pores and also helps in accelerating the crosslinking time of the hydrogels.

Directional Freezing of Liquid CO_2 to Create Aligned Porous Structures

Controlled freezing can be used to create aligned porous structures.[85,86] For example, Zhang *et al.*[87] introduced a novel technique to create an aligned porous structure with a sugar acetate (1,2,3,4,6-pentaacetyl β-D-galactose) material. Their approach was to first solubilize the sugar acetate in liquid CO_2 (75 bar) within a cylindrical column, which was then

slowly lowered into liquid nitrogen to freeze the CO_2. This directional freezing process was responsible for the creation of aligned tubular pores within the sugar acetate structure, which was recovered as a continuous monolith by simply subliming the CO_2 at ambient pressure. This interesting approach is of broad interest, including in the field of tissue engineering and beyond.

Use of Dense-Phase CO_2 for Polymer Sintering

Our group later formed microsphere-based scaffolds containing cells by using subcritical CO_2 sintering.[88] PLGA microsphere scaffolds were sintered using a subcritical CO_2 pressure of $\sim$15 bar at 25°C for 1 h, followed by depressurization at a rate of $\sim$0.14–0.21 bar/s. During subcritical CO_2 sintering, the equilibration of CO_2 in the polymer was restricted due to the short exposure time and low-pressure conditions, which led to a comparatively reduced plasticized state in the center of the spheres than that achieved during gas foaming. The microspheres retained their spherical shape during this process, and the slight swelling of the microsphere surfaces and subsequent adhesion (and possibly reptation) led to sintering of the adjoining microspheres, thereby resulting in a porous matrix. We applied this technology to form porous scaffolds that facilitated the growth of chondrocytes for cartilage tissue engineering applications. Cell viability during subcritical CO_2 sintering was also evaluated in this study. Human umbilical cord mesenchymal stromal cells at a density of 1×10^6 cells were mechanically mixed with microspheres and exposed to CO_2 at a pressure of 30 bar for 4 min, followed by depressurization at a rate of $\sim$0.2 bar/s. Viability tests revealed that almost the entire cell population survived the sintering process.

Incorporation of Growth Factors and Mammalian Cells

As discussed in the previous section, there are many methods utilizing supercritical fluid technology to prepare 3D scaffolds. While these scaffolds provide some degree of mechanical integrity and are biocompatible on implantation, they alone may not be sufficient to promote cell adhesion, proliferation, and differentiation into the desired tissue. They often require the presence of cell-signaling molecules and other bioactive compounds. Research has been conducted to encapsulate drug delivery molecules, bioactive signals and cells, and genes to promote cellular infiltration and differentiation using CO_2.

Hile *et al.*[89] were among the first to incorporate growth factors into polymeric foams using supercritical CO_2. They made porous poly(D,L-lactide-co-glycolide) containing basic fibroblast growth factor (bFGF) to promote angiogenesis. A homogenous water-in-solvent emulsion was prepared with the protein in the aqueous phase and the polymer in the organic phase. Saturating the emulsion with supercritical CO_2 followed by depressurization led to the formation of porous scaffolds encapsulated with the protein. The release rate of active bFGF from these porous scaffolds was not as high as that from salt-leached scaffolds, and there was greater solvent residue remaining. In a similar manner, drug delivery molecules can be encapsulated within a porous scaffold and used for cell culture.[90]

Duarte *et al.*[91] utilized a supercritical fluid impregnation method to prepare a chitosan scaffold containing dexamethasone. Loading of the bioactive compound was found to be most successful at a pressure of 80 bar and a temperature of 35°C, and increasing the pressure and temperature resulted in lower encapsulation efficiency. The dexamethasone release was relatively rapid, with $\sim$90% released within 2 h.[91]

Ennett *et al.*[92] explored the release kinetics of vascular endothelial growth factor (VEGF) from PLGA scaffolds after being incorporated via supercritical CO_2 foaming, both *in vitro* and subcutaneously in mice. They found that the method of VEGF incorporation had a greater effect on release kinetics than the polymer composition, and that local angiogenesis was significantly enhanced *in vivo*. Kanczler *et al.*[93] later encapsulated VEGF in PLA scaffolds by supercritical foaming, and seeded them with human bone marrow stromal cells. They found that the combination of temporally delivering VEGF from scaffolds seeded with human bone marrow mesenchymal stem cells (hBMSCs) resulted in enhanced bone regeneration of a mouse femur segmental defect. In a follow-up study from the same group,[94] a scaffold of alginate fibers embedded in PLA incorporated both VEGF and bone morphogenetic protein (BMP)-2, whereby the BMP-2 was encapsulated in the PLA along with VEGF-loaded fibers using supercritical CO_2. These constructs, seeded with hBMSCs, successfully regenerated bone in a mouse critical-sized femur defect.

Alternatively, microparticles and nanoparticles can also be used as carriers for bioactive compounds. Santo *et al.*[95] demonstrated the utility of this technique in impregnating $P_{DL}LA$ with chitosan/chondroitin sulfate nanoparticles. The scaffolds were fabricated by supercritical CO_2 foaming at 200 bar and 35°C. Homogeneous distribution of the nanoparticles was observed throughout the 3D scaffold. It was also noted that there was swelling (water uptake) of the construct due to the entrapment of the nanoparticles. The resultant scaffold was found to have adequate mechanical integrity, porosity, and pore interconnectivity for supporting cells. *In vitro* studies revealed that this system could be used as a promising candidate for dual protein delivery systems for potential applications in tissue engineering. In another study published that year, human growth hormone (hGH) was encapsulated in PLGA/PLA microspheres with supercritical CO_2.[96] Sustained hGH release was demonstrated in both rats and monkeys that could not be achieved with a single-soluble administration. Although this was not a tissue engineering application, this type of approach could readily be tailored to a tissue engineering strategy by encapsulation of any desired bioactive signal and either impregnating a scaffold (e.g., hydrogel) with these microspheres, or by sintering the microspheres together into a scaffold of any desired shape with ethanol[97–99] or even dense-phase CO_2.[88]

Supercritical fluid technology is also being used to explore DNA delivery in polymeric foams for potential applications in tissue engineering. Nie *et al.*[100] is one such group that made use of supercritical CO_2 for plasmid delivery. In their study, PLGA/chitosan foams were made by combining the techniques of spray drying with supercritical CO_2. PLGA microspheres encapsulated with plasmid DNA were prepared using spray drying. The microspheres were then combined with chitosan molecules to form foams using supercritical CO_2. A CO_2 pressure of 120 bar was used for a

period of 2 h, after which the pressure was reduced to ambient conditions at a rate of 0.5 bar/s. Sustained DNA release was observed from these scaffolds. The integrity of the plasmids was also found to be well maintained. While increasing the content of chitosan caused a decrease in the release rate of DNA, it proved to be helpful in facilitating cell adhesion and viability.

Processing of mammalian cells during supercritical CO_2 foaming of scaffolds was first tried by Ginty *et al.*[101] They developed a single-step supercritical CO_2 technique to prepare PLA scaffolds that contained a cell suspension. Various mammalian cell types such as a myoblastic C2C12 cell line, 3T3 fibroblasts, chondrocytes, and hepatocytes were investigated for their viability. Upon depressurizing, a polymer sponge containing viable cells was obtained. The functionality of C2C12 cells was demonstrated by their osteogenic response to the bioactive compound BMP-2. While this is a convenient one-step process, the time-dependent survival of cells poses a major challenge. To overcome this issue of cell viability, Ginty *et al.*[102] developed a high-pressure CO_2 injection port to deliver mammalian cells into an already plasticized scaffold during the foaming process. The cells were shown to be viable and were able to undergo osteogenic differentiation. In addition, the cells were able to retain both metabolic and enzyme activity.

CO_2 technology can hence be used to encapsulate a variety of compounds such as bioactive signals, drugs, and plasmids for gene delivery. In addition, CO_2 may also be used to incorporate cells into scaffolds as they are fabricated, performed in a single step.

Conclusions

The formation of scaffolds with desirable properties for tissue engineering applications remains a challenge. The conventional CO_2 foaming process has been developed extensively to prepare porous scaffolds with a high degree of porosity and pore interconnectivity from both natural and synthetic polymers. Process parameters such as temperature, pressure, depressurization rate, soaking time, venting time, and chemical properties of the polymer are governing factors for controlling the macro- and microarchitecture of the 3D construct, especially with regard to pore architecture and interconnectivity, with key examples established in the literature.[2,18,49,58,62]

Preparation of 3D constructs that are able to reproduce the highly complex spatial organization of cells and extracellular matrix as seen in complex tissues is the need of the day. As an example, Salerno *et al.*[57] used a concentration gradient of NaCl to create spatial gradients of pore size and porosity. In addition, our team has created opposing gradients in bioactive signal release,[97–99,103] material composition,[104] or both,[105] using microspheres, and microsphere-based scaffolds can be sintered together with CO_2,[88] so CO_2 can be used to create scaffolds with inherent gradients in composition and signal release.

A few key examples of exciting applications of CO_2 in tissue engineering include the use of supercritical CO_2 as a swelling agent for polymers to help impregnate the scaffold with desirable additives such as drugs and bioactive compounds, as well as using CO_2 as a compressed fluid to obtain different polymer morphologies at lower temperatures and pressures <100 bar. Another major finding is the use of CO_2 at much lower pressures to sinter together microspheres in the presence of cells. Perhaps the greatest contribution to the literature has been the advances of CO_2-foamed scaffolds, from early initial studies in developing classical nucleation theory for the CO_2 foaming of polymers, to pioneering studies in adapting this technology to tissue engineering that revealed challenges in pore interconnectivity and the skin layer, and to more advanced studies that have overcome these challenges, and thus made the technology far more accessible for the tissue engineering community to adapt.

The timing is perfect for the tissue engineering community as a greater whole to employ CO_2 as an outstanding tool to encapsulate growth factors, sinter polymers, and to foam scaffolds. With the low cost and ease of implementation, along with the advantage of circumventing the use of organic solvents, and now with the tremendous precedent that has been set with the operating conditions that should be used (and how they can be adjusted and tuned for new systems), the ground is fertile for the cultivation of a new generation of researchers to leverage CO_2 as a new tool to enhance their respective unique capabilities.

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