



Engineering porous scaffolds using gas-based techniques

Fariba Dehghani and Nasim Annabi

Scaffolds are used in tissue engineering as a matrix for the seeding and attachment of human cells. The creation of porosity in three-dimensional (3D) structures of scaffolds plays a critical role in cell proliferation, migration, and differentiation into the specific tissue while secreting extracellular matrix components. These pores are used to transfer nutrients and oxygen and remove wastes produced from the cells. The lack of oxygen and nutrient supply impedes the cell migration more than 500 μm from the surface. The physical properties of scaffolds such as porosity and pore interconnectivity can improve mass transfer and have a great impact on the cell adhesion and penetration into the scaffolds to form a new tissue. Various techniques such as electrospinning, freeze-drying, and solvent casting/salt leaching have been used to create porosity in scaffolds. The major issues in these methods include lack of 3D structure, control on pore size, and pore interconnectivity. In this review, we provide a brief overview of gas-based techniques that have been developed for creating porosity in scaffolds.

Address

School of Chemical and Biomolecular Engineering, The University of Sydney, Sydney 2006, Australia

Corresponding author: Dehghani, Fariba
(Fariba.dehghani@sydney.edu.au)

Current Opinion in Biotechnology 2011, **22**:661–666

This review comes from a themed issue on
Tissue, cell and pathway engineering
Edited by Uwe T. Bornscheuer and Ali Khademhosseini

Available online 3rd May 2011

0958-1669/\$ – see front matter
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DOI [10.1016/j.copbio.2011.04.005](https://doi.org/10.1016/j.copbio.2011.04.005)

Introduction

The fabrication of porous scaffolds with desirable properties for tissue engineering applications remains a complex and challenging process. The key requirements for scaffold fabrication techniques include control of macrostructures and microstructures, the maintenance of biocompatibility, and mechanical properties [1]. The pore architecture of scaffolds, including porosity and pore interconnectivity, and average pore size, is critical in cell survival, proliferation, and secretion of extracellular matrices (ECMs) [2,3]. Large pores allow effective

nutrient supply, gas diffusion, and metabolic waste removal, but lead to low cell attachment and intracellular signaling. Small pores, however, have opposite effects [4]. Consequently, the construction of scaffolds containing both macropores and micropores may provide the essential physical support for cellular growth. The effect of implant pore size on tissue regeneration is emphasized by experiments demonstrating the optimum pore size of 5 μm for neovascularization, 5–15 μm for fibroblast ingrowth, 20–125 μm for the regeneration of adult mammalian skin, 100–350 μm for the regeneration of bone, 40–100 μm for osteoid ingrowth, and 20 μm for the ingrowth of hepatocytes [5]. Fibrovascular tissues also require pore sizes greater than 500 μm for rapid vascularization and survival of transplanted cells [6].

Various methods including electrospinning, freeze-drying, and solvent casting/salt leaching have been used to generate porous scaffolds; however, the disadvantages of these techniques include the use of toxic organic solvent, the formation of thin 2D structures, non-homogenous and limited porosity, irregularly shaped pores, and insufficient pore interconnectivity [1,7]. Gas-based techniques have been employed to address some of the issues associated with the use of these conventional methods for porosity generation. The gas foaming process utilizes the nucleation and growth of gas bubbles (internal phase) dispersed throughout a polymer (continuous phase) [8]. The gas bubbles can be generated *in situ* either via chemical reaction [9,10^{••},11] or by the addition of an inert gas to the polymer phase at low [12,13] or high pressure [14[•],15]. Different gas-based techniques used to produce porous scaffolds are summarized in Table 1.

Conventional gas foaming

In gas foaming technique, a foaming agent such as sodium bicarbonate is added into the polymer phase to generate an inert gas such as N_2 or CO_2 at moderate acidic solutions. The porous structure of polymer is formed when the dispersed gas phase (discontinuous phase) is removed from the continuous phase of polymer. In this method, the fabricated polymeric foams have low kinetic stability; because of the large difference between the densities of the gas and liquid. The liquid phase tends to drain downwards while the gas tends to move upwards [10^{••},13], which leads to the formation of inhomogeneous foam with a non-porous bottom layer and highly porous top surface. A surfactant is commonly added to the polymer solution to stabilize the foam; it prevents liquid drainage which causes bubble coalescence [9]. The destabilization of the

Table 1

Summary of different gas-based techniques used to produce porous scaffolds.

Fabrication method	Polymer	Pore size (μm)	Reference
Gas foaming/salt leaching	PCL	158–540	[24]
	PLGA	10–90	[31]
		38.8–323.9	[42]
	PLA	34.1–59.7 ^a	[43]
		580.1	[42]
Gas foaming and selective polymer extraction (GF/PE)	PCL	38–312	[32 ^{**} ,33]
Foaming by the <i>in situ</i> generation of a gas	Alginate	100–300	[9]
	Gelatin	230	[10 ^{**}]
	PLLA	100–300	[17]
	PEGDA	100–600	[11]
	Acrylic acid (AA)-acryl amide	100–250	[44]
Foaming through the insufflating of an inert gas	Gelatin	250–360	[13]
	Hyaluronic acid	120	[12]
	Chitosan	250	[12]
	Alginate	206	[9]
CO ₂ -water emulsion templating	Dextran	6–25.7	[35 [*]]
	Chitosan, PVA, PVA/PEG	3–15	[40]
	Alginate	23.9–250	[23]
	CaCO ₃ /PAM	4.7–4.9	[37]
Dense gas CO ₂ and a cosolvent	Gelatin	80–120	[39]
Using high pressure CO ₂ during crosslinking	Elastin	79.8	[22]
	Chitosan	32–43.9	[41]
	Tropoelastin/elastin	78	[21]
	PCL/elastin	5–540	[24]

^a Gas foaming without the addition of salt was used to process the scaffold.

polymeric foams can also be minimized through: firstly, increasing the viscosity of solution [9]; secondly, using polymers which undergo fast solidification by temperature changes (i.e. gelatin) [10^{**}]; and thirdly, the addition of a crosslinker and initiator to the polymer solution to induce rapid polymerization after the generation of gas bubbles [11,16]. This technique has been used for creating porosity in polymers such as alginate [9], gelatin [10^{**}], poly(ethylene glycol) diacrylate (PEGDA) [11], and poly(L-lactic acid) (PLLA) [17].

Barbetta *et al.* prepared a porous structure of alginate foam by generating CO₂ through the reaction between tartaric acid and sodium bicarbonate in the presence of a suitable surfactant (i.e. Pluronic F-108) [9]. The foam was then frozen in liquid nitrogen, freeze-dried, soaked in a solution of CaCl₂ to form physical gel, and subsequently chemically crosslinked to form a stable hydrogel [9]. The resultant hydrogels consisted of interconnected pores in the range of 100–300 μm macropores and 30–80 μm micropores [9]. A similar approach was used to generate porous gelatin scaffolds containing both macropores (230 μm) and micropores (90 μm) [10^{**}]. In this study, N₂ gas was generated by the reaction between sulphamic acid and sodium nitrite, and a combination of sodium dodecyl sulphate and polyQ surfactants for foam stabilization [10^{**}]. Although the fabricated gelatin scaffold was shown to support hepatocyte adhesion and viability,

albumin and urea secretion was started to decrease after 15 days of culture time [10^{**}]. A highly interconnected, macroporous poly(ethylene glycol) diacrylate (PEGDA) hydrogel scaffold was also obtained by using sodium bicarbonate as a blowing agent, pluronic-F 127 as foaming stabilizer, and *N,N,N',N'*-tetramethylethylene-diamine initiator [11]. After the formation of gas bubbles, the polymerization of PEGDA was allowed for 30 min to produce PEGDA hydrogel, with pores ranging from 100 to 600 μm . This hydrogel was found to support the adhesion and long-term viability of human mesenchymal stem cells (hMSCs) and facilitated mineralization when exposed to osteogenic medium [11].

Porosity was created in the structures of biopolymers including gelatin [13], hyaluronic acid, chitosan, and alginate [12] by insufflation of an inert gas inside an aqueous solution of biopolymer in the presence of a suitable surfactant. In this method, the foam was generated through the injection of argon from a porous septum at the bottom of a reactor containing biopolymer solution and surfactant. The foam was then immediately frozen in liquid nitrogen, freeze-dried, and crosslinked to avoid foam dissolution. It was reported that the resultant scaffold had pore sizes in the range suitable for cell infiltration and proliferation (Table 1). For example, the fabricated gelatin scaffold had interconnected pores with sizes between 250 and 360 μm [13].

Conventional gas foaming techniques are efficient for the creation of porosity in water-soluble polymers that can form hydrogels. The main advantage of these techniques includes fabrication of highly porous scaffolds with interconnected pores. However, it is necessary to use surfactant and highly viscous solution for the creation of scaffolds. The residue of surfactant may have a negative impact on the biocompatibility of scaffolds. The use of not toxic surfactant (i.e. phospholipids) may extend the applications of conventional gas-based techniques for the fabrication of biocompatible scaffolds in tissue engineering.

Dense gas foaming

Gas foaming process using high pressure CO₂ has been employed for porosity generation in hydrophobic polymers [14[•],15,18,19], hydrophilic polymers [20^{••},21–23], and hydrophilic/hydrophobic composites [24^{••},25].

Porosity generation in hydrophobic polymers

There are three basic steps in gas foaming process: firstly, plasticization due to CO₂ diffusion into the polymer matrix at high pressure; secondly, nucleation of gas bubbles as a result of depressurization and supersaturation; thirdly, gas bubbles growth due to the gas diffusion from the surrounding polymer [15,26]. Skin layer formation and poor pore interconnectivity, which are common issues in most pore formation techniques [27[•],28,29], can be avoided in this method by the addition of salt particles to polymer matrix before gas foaming [30,31]. Porous structures of amorphous or semi-crystalline hydrophobic polymers such as poly(lactic) acid (PLA), poly(DL-lactic acid-glycolic acid) (PLGA), poly(ϵ -caprolactone) (PCL), poly(methyl methacrylate) (PMMA), and polystyrene have been obtained using gas foaming technique [14[•],15,18,19]. Salerno *et al.* used gas foaming process to produce PCL foams with porosity in the range of 78–93% and pore sizes between 10 and 90 μm [31]. Annabi *et al.* fabricated highly porous PCL scaffold with average pore sizes of 540 μm by using gas foaming/salt leaching process [24^{••}].

A combined gas foaming and selective polymer extraction (GF/PE) technique was designed to fabricate porous PCL scaffold with well-controlled microstructures [32^{••},33,34]. In this technique, PCL was first melt-mixed with thermoplastic gelatin (TG) at 60°C and then gas foamed by using a mixture of N₂/CO₂ as blowing agent at 70°C and 180 bar. The TG was subsequently removed by soaking the blend in water to form a porous structure of PCL contained both macropores ($D \sim 312 \mu\text{m}$), created by the removal of TG, and micropores that were formed during PCL foaming ($D \sim 38 \mu\text{m}$) [32^{••},33]. The fabricated porous PCL scaffolds supported hMSCs adhesion, proliferation, and osteogenic differentiation *in vitro* [32^{••}]. Dense gas foaming technique can be used for the creation of porosity in amorphous and semi-crystalline hydrophobic

polymers due to considerable solubility of dense gases such as CO₂ into these polymeric matrices, and their plasticization effects.

Porosity generation in hydrophilic polymers

Dense gas CO₂ generally has low solubility in hydrophilic polymers; consequently, gas foaming technique using high pressure CO₂ is not efficient for the creation of porosity in crystalline and hydrophilic polymers. Supercritical CO₂-water emulsion templating [23,35[•],36,37] and method that involves a co-solvent have been developed to improve CO₂ diffusion into a hydrophilic polymer [38,39]. In a recent study, a biodegradable polymer such as collagen or gelatin and a solvent such as ethanol or diluted acid were placed in a high pressure chamber [39]. The vessel was then pressurized at a desired temperature and pressure to allow a supercritical fluid dissolve into the polymer with the aid of the co-solvent [39]. Finally, the pressure was released and a porous structure was obtained. The pore size and morphology of the porous hydrogel can be controlled by adjusting the operating pressure and temperature [39]. Using this method, an open-pore structure of gelatine with pore sizes between 50 μm and 200 μm was generated. The main drawback of this method is the use of organic solvent during the process.

Supercritical CO₂-water emulsion templating was developed as a solvent-free process to produce highly porous hydrogels from various biopolymers such as dextran [35[•]], chitosan [40], and alginate [23]; also from synthetic polymers including poly vinyl alcohol (PVA), blended PVA/poly ethylene glycol (PEG) [40], and CaCO₃/polyacrylamide (PAM) composites [37]. CO₂-water emulsion polymerization templating was used to fabricate porous dextran hydrogels in the presence of perfluoropolyether (PEFE) as a surfactant and potassium peroxydisulphate (K₂S₂O₈) as an initiator for radical polymerization [35[•]]. The use of non-biodegradable surfactant and a mean pore size less than 26 μm may not allow this technique to be used for cell culture applications.

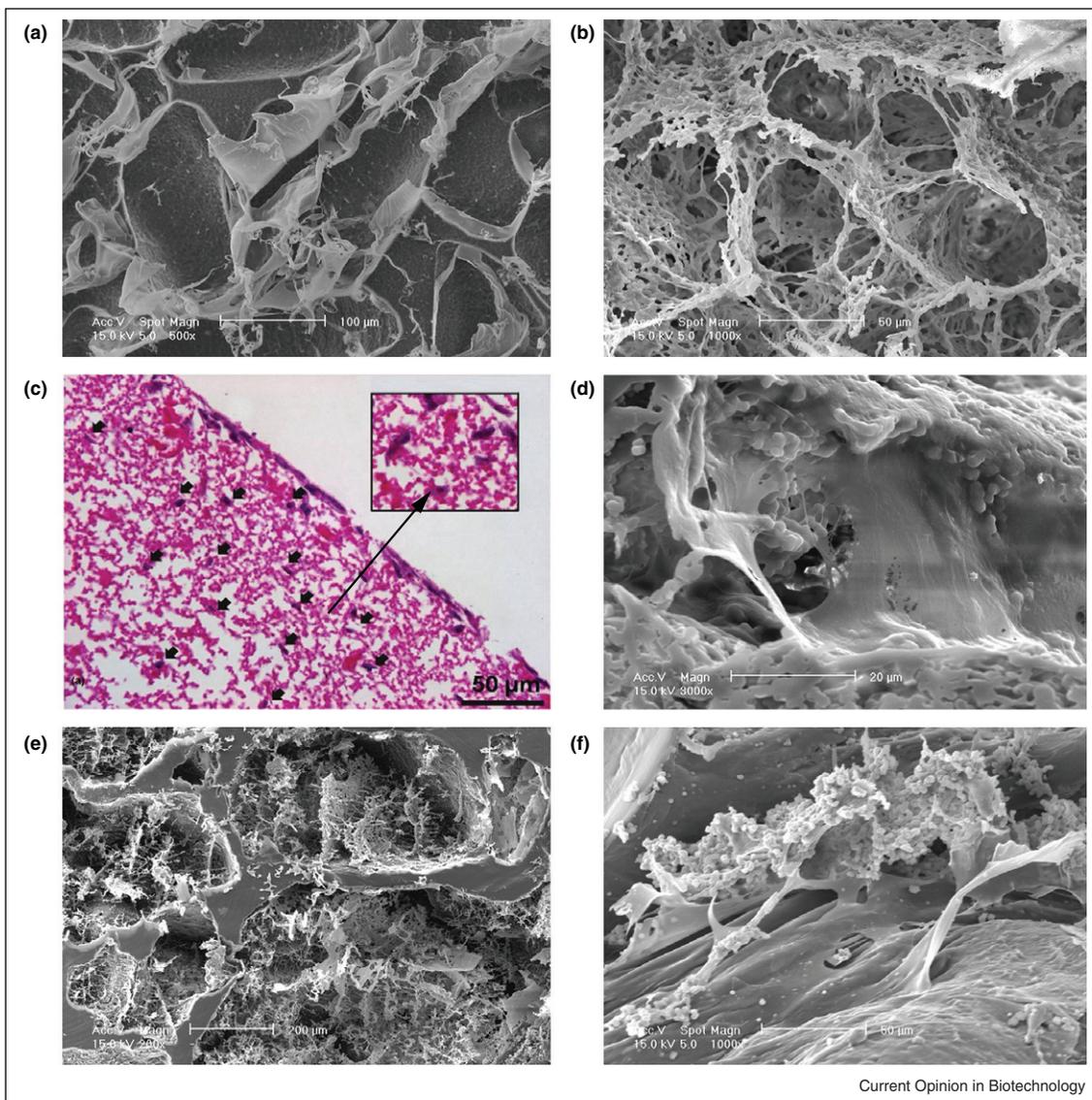
Cooper *et al.* synthesized a biodegradable and inexpensive poly (vinyl acetate)-based surfactant that can be used in supercritical CO₂-water emulsion templating to promote the product biocompatibility [40]. Porous crosslinked hydrogels from aqueous solution of PVA, blended PVA/PEG, and chitosan were synthesized at relatively low temperature and pressure (25°C and pressures less than 120 bar) by using a poly (vinyl acetate)-based surfactant. The resultant PVA hydrogel had highly interconnected pore structure with pore size in the range of 3–15 μm [40]. Supercritical CO₂-water emulsion templating technique was also proposed by Partap *et al.* to produce physically crosslinked alginate hydrogels [23]. In this approach, an aqueous solution of sodium alginate, calcium carbonate, and ammonium perfluoropolyether

(PFPE-NH₄) surfactant was exposed to 100 bar CO₂ pressure and 50°C. Supercritical CO₂ simultaneously served as the templating agent as well as inducing acidity to release calcium ions from their chelated form and induce physical crosslinking [23]. The fabricated porous alginate hydrogels displayed an interconnected pore network with a broad pore size distribution between 24 μm and 250 μm depending on the surfactant concentration and CO₂ fraction [23].

Annabi *et al.* developed a dense gas process to fabricate highly porous hydrogels from biopolymers including elastin [20,22], chitosan [41], and tropoelastin/elastin [21]. These hydrogels were formed at relatively low temperature and CO₂ pressure (37°C and pressures below 65 bar),

without using any surfactant. In this method the biopolymer solution is mixed with a crosslinking agent and the system is pressurized with CO₂ to dissolve the gas into the aqueous phase. Subsequent depressurization induces pores in the 3D structures of the hydrogels. As shown in Figure 1, high pressure CO₂ had a significant effect on pore morphology of fabricated hydrogels. In this method the skin-like formation was not observed on the top surfaces of hydrogels and large pores were created with an average pore size of $78 \pm 17 \mu\text{m}$ (Figure 1b) [21]. *In vitro* studies showed that the fabricated hydrogel supports human skin fibroblast growth and migration up to 300 μm into the 3D construct for elastin-based hydrogel (Figure 1c, d) [21] and up to approximately 50 μm for chitosan [41].

Figure 1



GA crosslinked tropoelastin/elastin hydrogels fabricated using atmospheric pressure (a), and (b)–(d) dense gas CO₂. PCL/elastin composite scaffold produced by using gas foaming/salt leaching process (e) and (f).

Porosity generation in hydrophobic/hydrophilic composites

Dense gas CO₂ has been recently used for porosity generation in hydrophilic/hydrophobic hybrid scaffolds such as poly (DL-lactic acid) (PDLLA)/chitosan/chondroitin sulphate nanoparticles (NPs) composites [25], and PCL/elastin composites [24**]. Santo *et al.* used supercritical CO₂ to fabricate hybrid 3D scaffolds of PDLLA loaded with chitosan/chondroitin sulphate nanoparticles for biomacromolecule delivery in tissue engineering [25]. In this study, NPs suspended in ethanol was added to PDLLA powder and pressurized with CO₂ in a high pressure vessel to 200 bar at 35°C [25]. The fabricated composites had porosity and pore interconnectivity of 56% and 39%, respectively, and displayed adequate mechanical properties (compression modulus of 11.3 MPa) for cell adhesion and support [25]. The feasibility of using this scaffold as a multifunctional material was evaluated by the incorporation of a model protein, bovine serum albumin (BSA), either directly into the PDLLA foam or in the NPs that were eventually included in the scaffold. It was reported that this composite could control the release of BSA as a model protein; therefore, the system was a promising candidate for dual protein delivery system for tissue engineering applications [25].

Annabi *et al.* developed a two-stage solvent-free dense gas technique to produce porous 3D structures of hydrophilic/hydrophobic composites [24**]. In the first stage, gas foaming/salt leaching process was used to create large pores with the average pore sizes of 540 ± 21 µm in a PCL matrix. The PCL scaffolds were then impregnated with elastin and crosslinked with glutaraldehyde (GA) under high pressure CO₂ to form microporous structure of cross-linked elastin within the macropores of PCL (Figure 1e) [24**]. The presence of elastin within the pores of PCL improved the water uptake properties of PCL scaffolds; the water uptake ratio of PCL was enhanced 100-fold, when elastin solution concentration of 50 mg/ml was used for composite preparation [24**]. The fabricated PCL/elastin composites exhibited compressive modulus that exceeded the target value for articular cartilage [24**]. *In vitro* studies showed that these composites could support primary articular cartilage chondrocyte adhesion and proliferation within the 3D structures (Figure 1f) [24**].

Conclusions

The microstructure of scaffolds has significant impacts on the cellular adhesion, proliferation, and secretion of ECM. Conventional gas foaming and dense gas foaming processes have been developed to fabricate 3D and porous scaffolds with high level of pore interconnectivity from both synthetic and natural polymers. These techniques addressed some of the issues in conventional pore formation methods, including the creation of 3D structures, homogenous porosity, and high pore interconnectivity. Conventional gas foaming techniques can

manipulate the porosity by controlling the surfactant and viscosity of solution. Process parameters such as temperature, pressure, depressurization rate, and salt concentration are governing factors for controlling microstructures of scaffolds in gas foaming processes. A desirable gas foaming technique is the one that eliminates the use of surfactant and organic solvent; it has also potential to create pores suitable for the specific tissue engineering application. Gas foaming techniques enable the formation of porous and thick cross-section scaffolds that can increase mass transfer of oxygen and nutrients into the scaffolds. However, from the results reported in the literature, the thickness that cell can penetrate into the scaffolds was still below 500 µm. Further development is required for the micro-architecture of scaffolds to enhance oxygen and nutrient diffusion and promote cell proliferation into the deeper thickness.

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