

Supercritical Gel Drying of Polymeric Hydrogels for Tissue Engineering Applications

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Tissue engineering (TE) is an emerging field aimed at repairing defective tissues and organ, instead of relying on conventional organ transplantation. Among the polymeric materials proposed for TE applications, the water-soluble polymers have been frequently used in form of hydrogels, that are a class of highly hydrated polymer materials. Nevertheless, hydrogel materials have poor mechanical properties; as a result, this scaffolding approach is seldom used for hard tissues applications. With the aim of overcoming this limitation, drying processes have been often applied to eliminate the water from hydrogels and apply the range of applications of the water-soluble polymeric scaffolds, but these techniques present several limitations. For this reason, in this work we proposed a supercritical gel drying method coupled with a water/solvent substitution step. PVA, Alginate and Chitosan hydrogels were processed and opportunely characterized from a macroscopic and microscopic point of view. Supercritical gel drying process confirmed to be effectiveness for the formation of polymeric scaffolds; in particular, we obtained stable 3-D structures with nanometric porous morphologies suitable for TE applications.

1. Introduction

Tissue engineering (TE) is an emerging field aimed at repairing defective tissues and organ, instead of relying on conventional organ transplantation. Indeed, many difficulties arise with direct transplantation, due to insufficient organ donors, rejection of the donor organ and pathogens transmission.

Numerous strategies currently used to engineer tissues depend on employing a material scaffold. These scaffolds serve as a synthetic extra cellular matrix (ECM) to organize cells into a three-dimensional architecture and to present stimuli, which direct the growth and formation of a desired tissue (Yang et al., 2001). Depending on the tissue of interest and the specific application, the required scaffold material and its properties will be quite different, but, all tissues have to simultaneously provide (Ma, 2004): 1) a 3-D structure similar to the tissue to be substituted; 2) a very high porosity with an open-pore geometry and suitable pore size; 3) nano-structural characteristics mimicking the extracellular matrix (ECM); 4) mechanical properties to maintain the predesigned tissue structure and support the specific loadings applied to the original tissue; 5) biocompatibility and a proper degradation rate; 6) absence/reduced inflammatory response.

Various techniques have been reported in the literature for the fabrication of biodegradable scaffolds (Cari Pavia et al., 2012), such as gas foaming, fiber bonding, solvent casting/particulate leaching, phase separation, freeze drying and 3D-printing, but these techniques suffer several limitations; particularly, it is very difficult to obtain simultaneously the macro, micro and nanostructural characteristics that have been previously described.

Some supercritical carbon dioxide (SC-CO₂) assisted processes have also been proposed and various techniques have been used (Reverchon et al., 2012): supercritical induced phase separation (SC-IPS) (Tsivintzelis et al., 2007), supercritical foaming (Mooney et al., 1996), supercritical gel drying combined with particulate leaching (Reverchon et al., 2008), electrospinning in SC-CO₂ (Lee et al., 2007). The general aim of SC-CO₂ assisted techniques is to improve the traditional TE processes, using the characteristic properties of SC-CO₂ to control scaffold morphology through the modulation of mass transfer

properties. An efficient solvent elimination can be obtained due to the large affinity of SC-CO₂ with almost all the organic solvents and short processing times are possible.

Among the polymeric materials proposed for TE applications, water-soluble polymers have been frequently used in form of hydrogels, that are a class of highly hydrated polymer materials (water content over 30% w/w) (Park et al., 1992). They are composed of hydrophilic polymer chains, which are either synthetic or natural in origin. The structural integrity of hydrogels depends on crosslinks formed between polymer chains via various chemical bonds and physical interactions. Hydrogels used in TE applications are typically degradable, can be processed under relatively mild conditions, have mechanical and structural properties similar to many tissues and to the ECM, and can be delivered in a minimally invasive manner (Lee et al., 2001). Because of the injectable feature of these kind of scaffolds, this approach is sometimes referred as injectable scaffolding or in situ tissue engineering. Nevertheless, hydrogel materials have poor mechanical properties; as a result, this scaffolding approach is seldom used for hard tissues applications. With the aim of overcoming this limitation, drying processes have been often applied to eliminate the water from hydrogels and amplify the range of applications of the water-soluble polymeric scaffolds. The most used technique is the freeze drying; it is suitable for a wide range of hydrogel materials, including natural (Jin et al., 2008) and synthetic hydrophilic polymers (Liu et al., 2006). However, this method encounters difficulty in precisely tuning pore size since the hydrogel architecture formed using this method are extremely sensitive to the kinetics of the thermal quenching process. Other problems associated with this technique are the low structural stability and generally weak mechanical properties of the fabricated materials. The freeze-drying process often results in the formation of a surface skin because the matrix may collapse at the scaffold–air interface due to the interfacial tension caused by solvent evaporation (Ho et al., 2004); in addition, freeze-drying is energy intensive and requires a relatively long processing time for complete removal of the solvent (Quirk et al., 2005).

Recently, a SC-CO₂ gel drying process has been tested to generate 3-D scaffolds (Reverchon et al., 2008), maintaining the macro and nano structure of the gel. SC-CO₂ forms a supercritical mixture with the organic solvent used to produce the gel that is eliminated maintaining zero surface tension inside the solid structure contained in the gel. This operation avoids the gel collapse. However, SC-CO₂ shows a very limited compatibility with water at the ordinary temperatures and pressures used in SC-CO₂ processing; for example, at 40 °C and 100 bar, water solubility is around 0.5 % (Sabirzyanov et al., 2002). Therefore, the described process is not applicable to polymeric hydrogels.

Thus, the aim of the present work is to test the supercritical gel drying process to polymeric hydrogels (Polyvinyl alcohol (PVA), Alginate and Chitosan). A different procedure will be attempted, in which a water/solvent substitution step is added to the process. PVA, Alginate and Chitosan dried gels will be also characterized from a macroscopic and microscopic point of view.

2. Materials and Methods

2.1 Materials

PVA, Sodium Alginate and Chitosan were purchased from Sigma-Aldrich (St. Louis, MO), Acetic Acid glacial (99.9% purity), Acetone (99.8% purity) and Calcium Chloride were obtained from Carlo Erba Reagenti (Rodano, Mi - Italy) and Carbon Dioxide (99% purity) was bought from SON (Società Ossigeno Napoli - Italy). Water was distilled using a laboratory water distiller supplied by ISECO S.P.A. (St. Marcel, Ao - Italy). All materials were used as received.

2.2 Apparatus

Samples were prepared in a home-made laboratory plant that mainly consists of a 316 stainless steel cylindrical high-pressure vessel with an internal volume of 80 mL, equipped with a high pressure pump (Milton Roy – Milroyal B, France) used to deliver the SC-CO₂. Pressure in the vessel was measured by a manometer (Mod. 0.25, OMET, Italy) and regulated by a micrometering valve (Mod. 1335G4Y, Hoke, SC, USA), whereas temperature was regulated using temperature controllers (mod. 305, Watlow, Italy). At the exit of the vessel a rotameter (Mod. D6, ASA, Italy) was used to measure the CO₂ flow rate.

2.3 Procedures

PVA gels were prepared according to the following procedure. Solutions with PVA concentrations of 10 and 20% w/w in distilled water were prepared, and then acetone was added as the antisolvent at a distilled water/acetone ratio of 0.5; the solution was stirred and heated at 50°C until it become homogeneous. The PVA solution with distilled water and acetone was cooled at around -10°C and then a hydrogel was

obtained. Subsequently, water was substituted with an organic solvent (acetone) at the same temperature of gel formation (-10°C) for 24 h.

Sodium Alginate gels were prepared according to the following procedure. Calcium alginate sample were prepared by immersion of 2 % w/w of sodium alginate solution in a solution of calcium chloride at 5 % w/w, maintaining the temperature at 4°C . After 1h stirring, the resulting gel samples were stored in the coagulating bath for 1 h at room temperature. Sodium alginate reacted with calcium chloride to form samples and cross-linked calcium alginate was formed. The resultant samples were stored after washing with de-ionized water to remove CaCl_2 from the sample surface. Once produced the hydrogel, distilled water was replaced with an organic solvent; in particular ethanol or acetone, using a substitution bath at ambient temperature for 24 h.

Chitosan solution was prepared by dissolving chitosan (ranging between 5 and 20% w/w) in a water/acetic acid solution; the solution was stirred at 100 rpm and heated at 50°C until become homogenous. Then, the solution was poured in steel containers with a internal diameter of 2 cm and height of 1 cm and was frozen at a temperature of -20°C for 24 h to obtain an hydrogel. The hydrogel was put in a bath of acetone at -20°C for 24 h.

SC- CO_2 gel drying was performed on all samples according to the following procedure: the vessel was closed and filled from the bottom with SC- CO_2 . When the required pressure and temperature were obtained (200 bar and 35°C), drying was performed with a SC- CO_2 flow rate of about 1 Kg/h, that corresponds to a residence time inside the vessel of about 4 min; the drying lasted 4 h. A depressurization time of 20 min was used to bring back the system at atmospheric pressure.

2.4 Characterizations

Scanning electron microscopy (SEM). The samples were cryofractured using liquid nitrogen; then, they were sputter coated with Gold (Agar Auto Sputter Coater mod. 108 A, Stansted, UK) at 30 mA for 180 s and analyzed by a scanning electron microscope (SEM mod. LEO 420, Assing, Italy) to evidence the micro and nanostructure and to measure the diameter of the fibers forming the structure.

3. Results and Discussion

3.1 PVA

PVA is known for its excellent weight-bearing properties and biocompatibility (Lee et al. 2009). Recent work has investigated the potential role PVA hydrogels for tissue engineering of the articular cartilage in the knee (Stammen et al. 2001).

Supercritical drying process was attempted on PVA hydrogels prepared as reported in the procedures paragraph. The starting size and shape of the hydrogel samples were preserved during the supercritical drying step, confirming that the absence of surface tension at supercritical conditions allowed to generate stable 3-D structures. SEM images reported in Figure 1 show PVA structures obtained starting from different polymer concentration: 10 % w/w (Figure 1a) and 20 % w/w (Figure 1b). The presence of a very regular nanometric network structure is visible for both samples. But, the networks are different: nanometric fibers of about 500 nm (Figure 1a) for 10 % w/w PVA and nanometric cellular pores of about 100 nm (Figure 1b) for 20 % w/w PVA.

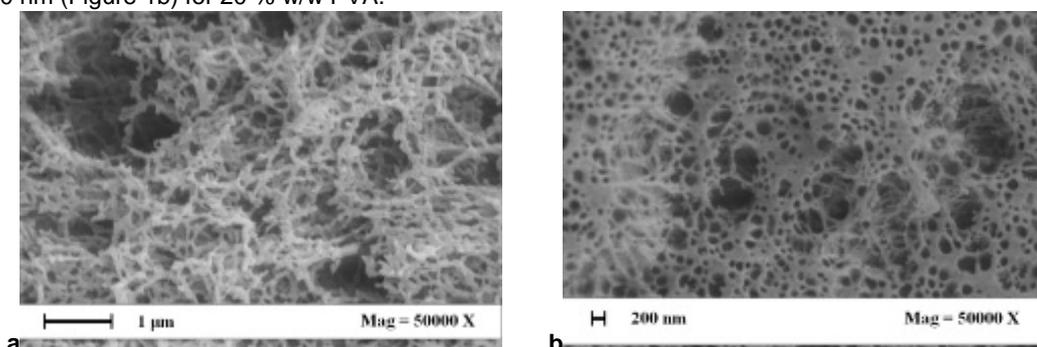


Figure 1: SEM images of the scaffolds obtained with supercritical gel drying at different PVA percentage: a) 10 % w/w of PVA, b) 20 % w/w of PVA

Both networks can be considered suitable from a tissue engineering point of view; indeed, cellular adhesion, growth, migration and differentiated function should be assured by the morphologies generated. But, it is also interesting to understand the reason of the change in the structural characteristics. The procedure proposed provides a freezing step at $-10\text{ }^{\circ}\text{C}$, during which the solution (PVA-Water-Acetone) is thermally phase separated (TIPS) and the structure is formed. The substitution of the water with acetone and the subsequent supercritical drying do not affect the morphology, but are useful to eliminate the water and generate the dry samples. For these reasons, the structural modification observed in figure 1 is due to the thermodynamic behavior of the system PVA-Water-Acetone during the TIPS step; in particular, at 10 % w/w of PVA, a bicontinuous fibrous structure is generated, typical of a liquid-liquid spinodal phase separation (Van de Witte et al., 1996), whereas, in the case of 20 % w/w PVA, a cellular structure is obtained, typical of a liquid-liquid binodal phase separation (Van de Witte et al., 1996). This result can be related to the shape of the miscibility gap in the diagram Temperature vs Polymer Concentration and to the different "pathways" of the solutions (depending on the starting polymer concentration) (Van de Witte et al., 1996): when the polymer concentration increases, the possibility of obtaining a binodal phase separation increases too, i.e., cellular structures are generated.

3.2 Sodium Alginate

Alginate is a naturally occurring polymer from brown algae. Due to cast stability at room/body temperature and construct porosity, alginate is an attractive material for scaffolds fabrication. Culture of MSCs in 3-D alginate beads showed high cell viability with little cellular proliferation. Upon dissolution of the alginate construct, MSCs returned to monolayer cultures of cells (Walker et al., 2009). In addition, culture of newborn rat hepatocytes in a macroporous alginate scaffold promoted maturation into functional hepatic tissue (Walker et al., 2009).

The samples were generated following the procedure proposed before, varying the water-substitution media (acetone or ethanol). SEM images reported in Figure 2 show the presence of a very regular nanometric network structure in the samples, either with the substitution water-ethanol (Figure 2a) either with the substitution water-acetone (figure 2b).

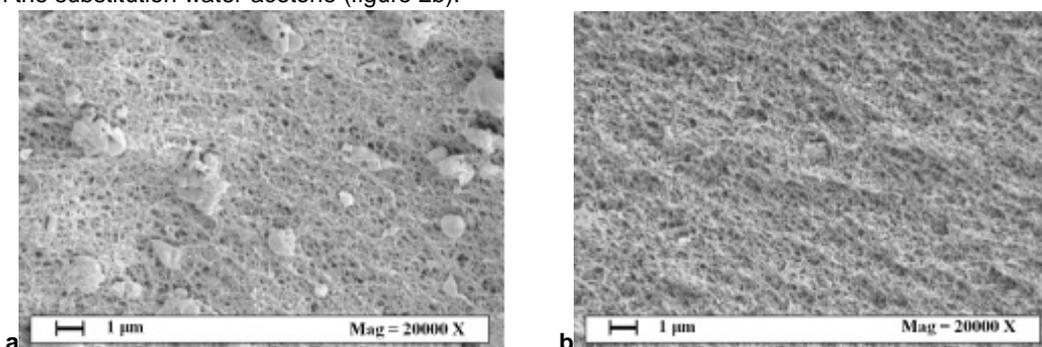


Figure 2: SEM images of the structure obtained with supercritical gel drying at 2% w/w of Sodium Alginate: a) with substitution water-ethanol, b) with substitution water-acetone

In these images, it is possible to observe that the fibrous nanometric network is uniform for both samples and the kind of substitution media used does not affect the final morphology. Also these networks ensure the structures "roughness" which promotes the cellular adhesion, growth, migration and differentiated function. As in the case of PVA, it is also possible to process more complex 3-D structures (Figure 3) confirming the possibility of producing samples with a specific geometry, without any collapse.



Figure 3: Sodium alginate 3-D structure obtained by SC-CO₂ drying

3.3 Chitosan

Chitosan is a linear polysaccharide with pro-coagulant properties derived from shrimp and crab shells. Classically, chitosan based bandages have been used for homeostasis in cases of acute hemorrhages (Englehart et al., 2008). Chitosan is insoluble in water but soluble at pH values under 6.5 in most acidic media. This polymer has been investigated extensively for several pharmaceutical field such as development of various controlled drug delivery. However recent work has investigated the potential role of chitosan based constructs for tissue engineering. Culture of both adipose tissue derived stem cells (ADSCs) and MSCs on chitosan scaffolds showed progenitor cell adhesion, proliferation and differentiation (Moreau et al., 2009).

The samples were generated following the procedure proposed before. In figure 4a, a SEM image of chitosan scaffold obtained starting from 10% w/w chitosan in water:acetic acid=98:2 solutions is reported. In these images, it is possible to observe that structures are completely uniform and are characterized by a nanometric fibrous network, with fibers of about 100 nm.

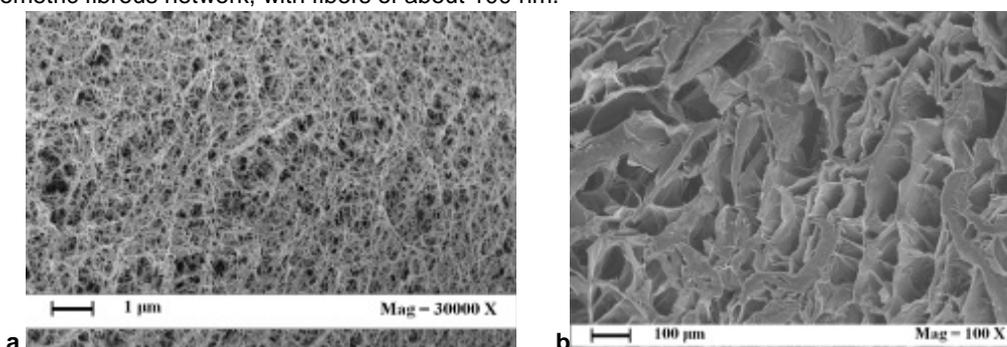


Figure 4: SEM images of 10% w/w Chitosan scaffold obtained starting from 2% w/w acid water solution (a) and starting from 5% w/w acid water solution (b)

We analyzed the effect of the percentage of acetic acid in the water (from 2 to 5% w/w). In Figure 4b, a SEM image of chitosan scaffold obtained starting from a water:acetic acid= 95:5 solution is reported; it shows the presence of a regular micrometric cellular structure with pores of about 80 μm, that is completely different from the nanofibrous network obtained with 2 % of acetic acid (figure 4a). This result can be explained considering again the thermodynamic behavior of the system. Changing the acetic acid content, the miscibility gap of chitosan/water/acetic acid system is modified; probably, the presence of a higher amount of acetic acid favored a binodal demixing, leading to the formation of a cellular structure; otherwise, lower acetic acid contents lead to a spinodal decomposition and the consequent formation of a fibrous bicontinuous structure.

4. Conclusions

Supercritical CO₂ drying method confirmed to be effectiveness for the formation of polymeric scaffolds starting from gel; in particular, during this work, we showed as it is possible to apply this process to

hydrogels too. We obtained stable 3-D structures with nanometric porous morphologies, suitable for tissue engineering applications.

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