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Natural Aerogels Production by Supercritical Gel Drying

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Aerogel-based scaffolds are frequently used for tissue engineering applications, since their nanofibrous structure is suitable for cells adhesion, proliferation and migration. Nevertheless, to achieve proper physico-chemical characteristics and desired mechanical properties, can be necessary to use more polymers together; among them, natural polymers are preferable because they are biocompatible, non-toxic, biodegradable and mucoahesive. In this study, bi-component natural aerogels Alginate-Gelatin (A/G) and Chitosan-Gelatin (CS/G) have been produced by Supercritical gel drying.
The results indicate that both A/G and CS/G mixtures formed uniform gels during the preparation step and Supercritical gel drying preserved their delicate nanostructured morphology, thanks to the near zero surface tension of supercritical mixture (CO_2 + organic solvent) formed during the drying process, as observed from FESEM pictures. All the aerogels were characterized by porosity values higher than 80%. Moreover, CS/G aerogels crosslinked with Glutaraldehyde (GTA) showed improved thermal behavior increasing gelatin content inside the starting hydrogel.

1. Introduction

Tissue Engineering (TE) is an innovative field born from the collaboration of engineers, biologists and clinicians, to overcome the problems of the traditional transplantation, in particular insufficient number of organ donors. It is aimed at regenerate tissues and organs of the human body using cells, active molecules (i.e., growth factor, proteins) and scaffolds (Reverchon and Cardea, 2012). Scaffolds are temporary supports, biodegradable and biocompatible, that, whether correctly designed, allow sufficient transport of gases, nutrients, and regulatory factors to the cells, favouring their survival, proliferation, and differentiation (Dhandayuthapani et al., 2011). To satisfy this aim, scaffolds have to be, simultaneously, micro and nano-structured, with high interconnection between the pores, and characterized by a 3-D shape similar to the tissue that they would regenerate (Ma, 2004).
Moreover, also the kind of material influences the scaffold performance. Synthetic polymers are highly useful in biomedical field since their properties (e.g., porosity, degradation time and mechanical characteristics) can be tailored for specific applications. Nevertheless, it is often necessary to modify their surface with specific molecules (e.g., Arg–Gly–Asp (RGD) peptide sequence) to allow cells inoculation and adhesion on the substrate (Evangelista et al., 2007). Natural polymers are biodegradable and biocompatible, and have better interactions with the cells with respect to the synthetic polymers, enhancing the cells' performance in biological system (Dhandayuthapani et al., 2011).
Generally, natural polymers are used in form of hydrogel and, since they are capable of retaining large amounts of water, together with their soft and rubbery consistence, they closely resemble living tissues (Van Vlierberghe et al., 2011).
Starting from hydrogel, freeze drying is the technique frequently used to obtain an aerogel. It is based on thermally induced phase variation of the solute due to solid-liquid demixing. Lowering of temperature below freezing point of the solvent, induces phase separation of the solution into a solvent phase and a polymer rich phase. Nevertheless, the obtained structure could collapse at nanometric level and mechanical properties are poor.

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To overcome these difficulties, supercritical assisted processes have been proposed in the pharmaceutical (García-González et al., 2011) and biomedical field for scaffolds generation such as, PCL (Karimi et al., 2012), PMMA (Cardea et al., 2014a), PLLA (Cardea et al., 2014b), PLLA loaded with ibuprofen (Cardea et al., 2014c) and Ethylcellulose (De Marco et al., 2014). Operating in this manner, it is possible to produce, in short processing time, nano and micro-porous solvent free polymeric structures, due to the negligible surface tension of the supercritical mixture (CO_2 + organic solvent). Cardea et al. (2014) tested the supercritical gel drying process on polymeric hydrogels (Polyvinyl alcohol (PVA), Alginate and Chitosan), obtaining stable 3-D structures with nanometric porous morphologies, with a mean fibre size from 500 nm for PVA sample, to 100 nm for chitosan sample, suitable for TE applications (Cardea et al., 2013). Reverchon et al. used the same process for the formation of poly(L-lactic acid) (PLLA)/hydroxyapatite (HA) scaffolds to improve the biomimethism and the mechanical properties of the scaffolds. They produced solvent free scaffolds (dioxane residue lower than 5 ppm) with a porosity higher than 90 % and an internal structure formed by nanofibers of 200 - 400 nm diameter. The loading of HA nanoparticles inside the structure produced a sensible increase of the compressive modulus of the PLLA scaffolds; in particular, it increased from 81 kPa for the scaffolds produced using PLLA alone, to 123 kPa when 50 % w/w of HA was loaded inside the scaffolds (Reverchon et al., 2009). Baldino et al. proposed a Supercritical Freeze Extraction Process (SFEP) that combines the Thermally Induced Phase Separation (TIPS) process with the supercritical drying, producing a complete and fast solvent elimination, avoiding the structure collapse. The authors obtained 3-D chitosan scaffolds characterized by a homogeneous microstructure, suitable for cells colonization and growth, and by a nanoporous sub-structure for cells interaction and guidance for adhesion, migration and differentiation. In particular, increasing from 5 to 10% w/w the polymer concentration, the scaffold mean pore size decreased from about 45 to 17 μm and pore walls showed nanofibers of about 80 nm diameter (Baldino et al., 2014).
The aim of this work is to use the supercritical gel drying (SC-gel drying) to produce alginate-gelatin and chitosan-gelatin bi-component natural aerogels for TE applications, and, since this process is not directly applicable to polymeric hydrogel, a water-ethanol exchange step will be performed to substitute ethanol to water, obtaining an alcogel, before the process. To determine the aerogels properties after the supercritical assisted process, they will be analyzed by a macroscopic and microscopic point of view.

2. Experimental section

2.1 Materials

Sodium Alginate, Gelatin from bovine skin (type B, bloom 225 g), Chitosan (Low Mw, deacetylation 75 – 85 %, viscosity 20 - 300 cps), Glutaraldehyde solution 25 % w/w in water and Calcium Chloride were bought from Sigma-Aldrich; Acetic Acid glacial (99.9 % purity) was bought from Carlo Erba Reagenti (Rodano, Mi - Italy); distilled water was produced using a laboratory water distiller supplied by ISECO S.P.A. (St. Marcel, Ao - Italy); CO2 (99 % purity) was purchased from S.O.N. (Società Ossigeno Napoli, Italy). All materials were processed as received.

2.2 Aerogel preparation

Alginate-gelatin (A/G) hydrogels were prepared dissolving 5 % w/w of each biopolymer in water; then, three samples in a volume ratio of 80/20, 50/50 and 20/80 (A/G) were mixed and poured in steel containers with an internal diameter of 2 cm and height of 0.5 cm. A/G samples were immersed in an aqueous solution of calcium chloride ($CaCl_2$) at 5 % w/w, and stored in the coagulating bath for 1 h at room temperature. The resultant hydrogels were washed for three times with de-ionized water to remove $CaCl_2$ from the sample surface. Subsequently, water was replaced with an organic solvent; in particular, ethanol, using a substitution bath at room temperature for 24 h, obtaining an alcogel. This step was necessary due to the low affinity of water with SC-CO_2 (Diamond and Akinfiev, 2003).
Chitosan-gelatin (CS/G) hydrogels were prepared dissolving 5 % w/w of each biopolymer in a water/acetic acid solution (pH 2.45); the solutions were stirred at 100 rpm and heated at 50 °C until it became homogenous. Three samples in a volume ratio of 80/20, 50/50 and 20/80 (CS/G) were mixed and poured in steel containers with an internal diameter of 2 cm and height of 0.5 cm. Then, samples were crosslinked with 2 mL of an acid water solution plus glutaraldehyde (GTA) at 1% v/v, under Vortex shaking for 1 min. Also in this case, acid water was replaced with ethanol, using a substitution bath at room temperature for 24 h.

2.3 Apparatus

Aerogels were prepared in a home-made laboratory plant that consists of a 316 stainless steel cylindrical high-pressure vessel with an internal volume of 80 mL, equipped with a high pressure pump (mod. LDB1, Lewa,

Germany) used to deliver the supercritical CO_2 (SC-CO_2). Pressure in the vessel was measured by a manometer (mod. MP1, 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 CO_2 flow rate.
SC-gel drying was performed on all alcogels according to the following procedure: the vessel was closed and filled 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 of about 4 min; the drying lasted 5 h. A depressurization time of 25 min was used.

2.4 Field Emission Scanning Electron Microscopy

Aerogels were cryo-fractured using liquid nitrogen; then, they were sputter coated with gold (Agar Auto Sputter Coater mod. 108 A, Stansted, UK) at 30 mA for 160 s and analysed using a Field Emission Scanning Electron Microscopy (FESEM, mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany.

2.5 Scaffold porosity

The porosity (ε) represents the “void space” of the sample and was calculated from the density of the aerogel and the density of untreated polymers. The aerogel density was determined by measuring its volume and weight and considering the volume ratio between the polymers; in particular, the volume was obtained using the Archimede’s principle. The sample was waterproofed and subsequently immersed in pure water; calculating the weight of the displaced water, we evaluated the volume of the sample.

2.6 Solvent residue analysis

Ethanol residues were measured by a headspace (HS) sampler (mod. 7694E, Hewlett Packard, USA) coupled to a gas chromatograph (GC) interfaced with a flame ionization detector (GC-FID, mod. 6890 GC-SYSTEM, Hewlett Packard, USA).

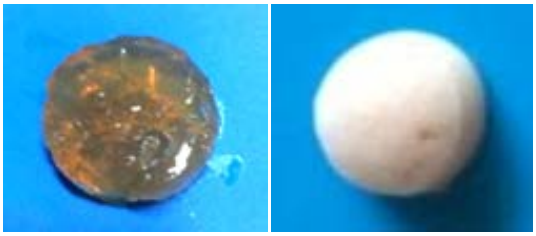
2.7 Thermogravimetric analysis

The thermal behaviour of aerogels was examined by thermogravimetric analysis (TGA, Q600, TA Instruments, Milano, Italy), online connected to a quadrupole mass detector (Quadstar 422, Pfeiffer Vacuum, USA) in Nitrogen atmosphere at a scanning rate of 10 K/min.

3. Results and Discussion

3.1 Alginate-Gelatin aerogel

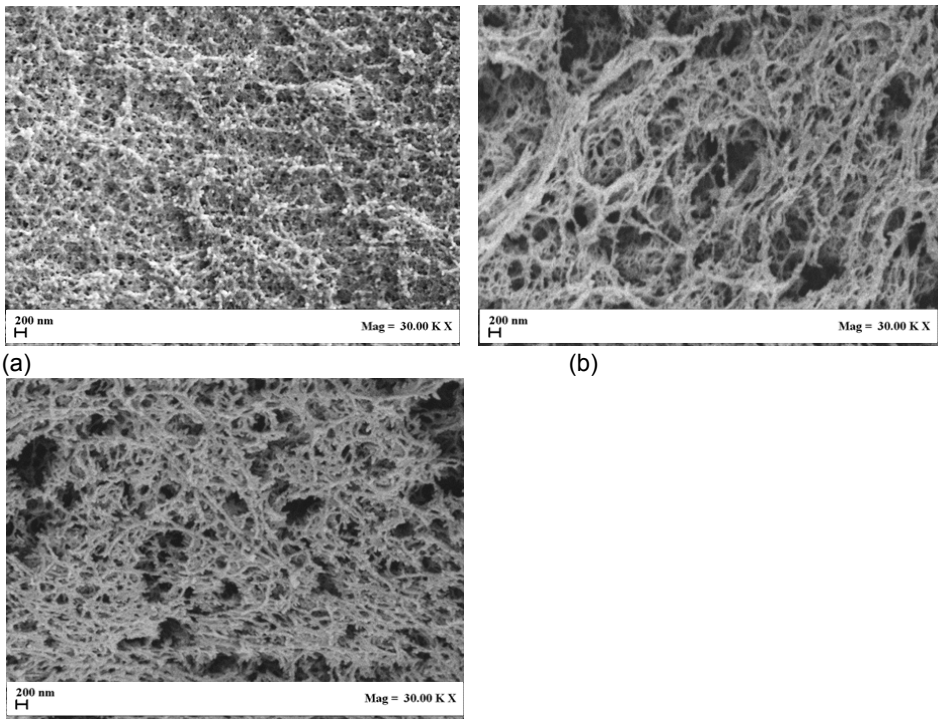
Alginate is a natural polymer, that can form structures similar to the tissues extracellular matrix (ECM); moreover, it becomes a gel under mild conditions and has low toxicity. But, it lacks cellular interaction; i.e., cells are unable to adhere to the alginate network. For this reason, we decided to add Gelatin to Alginate solutions, since it contains the Arg–Gly–Asp (RGD)-like sequences of amino-acids that promote the adhesion and migration of cells (Chandrasekaran et al., 2011).
Samples with A/G volume ratios of 80/20, 50/50 and 20/80 were prepared according to the procedure described in the Section 2.2 and processed by SC-gel drying at 200 bar, 35 °C for 5 h. The first observed result was the homogeneity of the biopolymeric solutions; i.e., when Alginate and Gelatin were mixed together in the different volume ratios, the resultant hydrogels and, the corresponding alcogels, were perfectly integrated and no separation phenomena were noted (Figure 1a). Samples maintained their shape and volume after the Supercritical drying (Figure 1b).



(a) (b)

Figure 1: (a) 50/50 A/G alcogel; (b) 50/50 A/G aerogel.

In Figure 2, FESEM pictures of A/G aerogels are reported. An uniform and regular morphology was obtained; this evidence confirms that during the process a SC-mixture (CO_2 + ethanol) was formed, that avoided structure collapse, since the organic solvent extraction was carried out at near zero surface tension.



(a) (b)

(c)

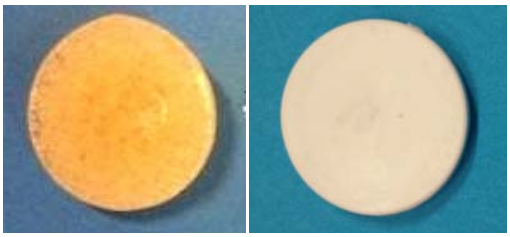
Figure 2: FESEM pictures of A/G aerogels 5 % w/w processed at 200 bar, 35 °C for 5 h: (a) A/G 80/20 (% v/v); (b) A/G 50/50 (% v/v); (c) A/G 20/80 (% v/v).

Moreover, we observed that increasing the Gelatin content in the starting hydrogel, aerogel with a nanofibrous morphology was obtained, with a mean fiber size of about 100 nm (Figure 2b-c). Instead, when Alginate volume content was higher (Figure 2a), a nanoporous structure was produced, with a mean pore size of about 80 nm. These results suggest that increasing Gelatin amount in the sample, the final morphology is similar to the one of the simple Gelatin, that is generally nanofibrous [Liu et al., 2009] and *viceversa*. Aerogels surface was open, whereas, using freeze drying, closed surfaces are often produced. The nanometric organization is mandatory for cells cultivation, since it mimics the natural ECM, and it is necessary for cells adhesion and migration on the support.
We also measured the scaffold porosity, and values of 81, 87.5 and 92% were found for A/G aerogels 80/20, 50/50 and 20/80, respectively. These results are consistent with the aerogels morphology variation from nanoporous to nanofibrous increasing Gelatin amount inside the hydrogel, as observed from FESEM images. Solvent residue analyses reported values of ethanol lower than 5 ppm for all the tested aerogels; i.e., as expected, SC-gel drying, at the selected operative conditions, allowed to completely remove the organic solvent from the aerogel.

3.2 Chitosan-Gelatin aerogel

Chitosan is biocompatible, biodegradable, osteoconductive and has an intrinsic antibacterial activity (Baldino et al., 2014). Also in this case, gelatin was added to chitosan solutions to enhance the final interactions between the scaffold and the cells; moreover, in the literature is reported that gelatin is a polymer that induces chitosan ionic gelation, due to the electrostatic interaction between the anionic charges of gelatin and the cationic charges of chitosan, forming a physical cross-linking (Dash et al., 2011).Samples with CS/G volume ratios of 80/20, 50/50 and 20/80 were mixed and cross-linked with GTA; after the acid water exchange with ethanol, they were processed by SC-gel drying at the same operative conditions of the previous experiments.

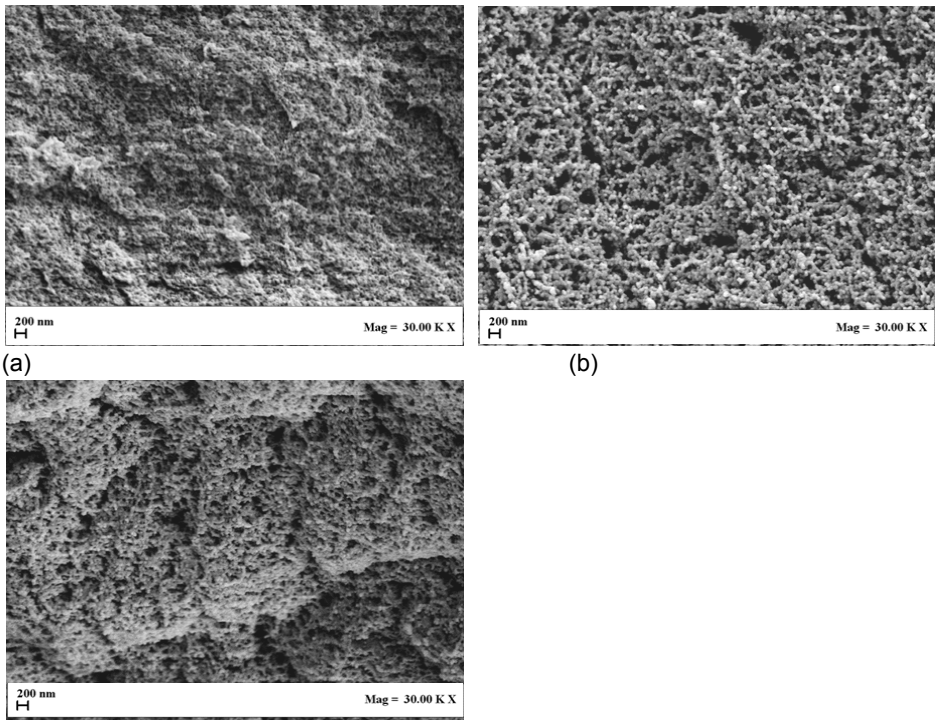
CS/G hydrogels were stable and homogeneous after the mixing and after the solvent substitution (Figure 3a). The supercritical assisted process preserved aerogel shape and volume, as observed in Figure 3b, in which 50/50 CS/G aerogel example is reported.



(a) (b)

Figure 3: (a) 50/50 CS/G alcogel; (b) 50/50 CS/G aerogel.

From FESEM analyses showed in Figure 4, we can observe that a nanoporous morphology was obtained in all samples tested. In particular, a mean pore size less than 100 nm was measured.



(a) (b)

(c)

Figure 4: FESEM pictures of CS/G aerogels 5 % w/w processed at 200 bar, 35 °C for 5 h: (a) CS/G 80/20 (% v/v); (b) CS/G 50/50 (% v/v); (c) CS/G 20/80 (% v/v).

Therefore, it was confirmed that Chitosan formed a gel due to the gelatin addition; indeed, in systems in which Chitosan alone was processed, it generally showed a microporous structure (Baldino et al., 2014). The presence of a matrix organized at nanometric level assures cells to be in direct contact with it, avoiding cells wash out during the dynamic culture step inside a bioreactor.
Porosity was 80, 86 and 91% and TGA showed a degradation temperature of 280, 287 and 305 °C for CS/G aerogel 80/20, 50/50 and 20/80, respectively. These results confirm the effect of GTA crosslinking in terms of aerogel improved stability. Moreover, the degradation temperature was higher for 20/80 CS/G aerogel, probably due to the different number of $–NH_2$ free amino groups not protonated in Gelatin with respect to Chitosan, that reacting with GTA, enhanced aerogel thermal behavior.
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4. Conclusions and perspectives

In this work, bi-component natural aerogels A/G and CS/G were successfully produced by SC-gel drying, preserving their delicate organization at nanoscale. This aerogels morphology, together with their good porosity and physico-chemical characteristics, could improve cells adhesion and migration and, generally speaking, enhance cells performance for TE applications.
In the following, it could be added a porogen to aerogels obtaining also a microporosity useful for nutrient and waste transport inside and outside the biological system, respectively, and to test their mechanical properties.

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