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Cu-alginate and Ca-alginate aerogels production by supercritical gel drying for biomedical applications

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ABSTRACT
In this work, alginate aerogels with a cylindrical shape were produced by supercritical drying for potential vascular application. In the first part of the work, using an *ad hoc* extruder, alginate (Alg) hydrogels at different polymer concentrations (from 5% to 15% w/w) were generated in a coagulation bath of calcium chloride. These cylindrical Ca-Alg-hydrogels were immersed in ethanol to induce water replacement with it, obtaining Ca-Alg-alcogels that were, subsequently, processed by supercritical drying at 200 bar and 45 °C for 4 h. Scanning electron microscopy showed that the delicate nanostructured morphology of the native Ca-Alg-hydrogels was preserved also after supercritical drying, confirming that at these operative conditions, a supercritical mixture (CO_2/EtOH) was formed with a negligible surface tension, that avoided gel collapse. Ca-Alg-aerogels fiber mean diameter was around 200 nm. BET analysis revealed that the Ca-Alg-aerogels specific area increased from 201 to 296.5 m^2/g when Alg concentration decreased from 15 to 5% w/w.
In the second part of this work, Alg-hydrogels were crosslinked with copper sulphate, selected for its angiogenic and antibacterial properties. The obtained Cu-Alg-aerogels were characterized by a nanoporous morphology with a maximum measured specific area of 111 m^2/g. UV/Vis spectrophotometer tests on Cu-Alg-aerogels revealed that the amount of unreacted Cu and released in a phosphate buffer medium, was between 98 and 160 ng; this Cu quantity was useful for blood vessels formation.

INTRODUCTION
The new frontiers of medicine for the regeneration of human tissues, are the stimulation in situ of the tissue to be regenerate or to implant functional engineered tissues [1]. In this last case, a crucial step is the selection of a biomaterial with proper chemical and physical properties. Generally, it should be biocompatible, biodegradable and of natural origin to minimize undesired human body reactions. Among the natural polymers frequently used, chitosan, gelatin and alginate are the most interesting since they are also cheap [2].
Other relevant aspects that positively influence tissue regeneration are the scaffold macroscopic shape similar to the tissue it would replace and the scaffold morphology at micro and nanoscale to facilitate cells adhesion, proliferation and differentiation in the specific tissue [3].
Several papers in the literature have been published in this field [4-7]; however, the main drawbacks are scaffold physical properties that also depend on the methodology used to produce them. In particular, scaffolds are generally produced by solvent evaporation, freeze drying, phase separation [1]. These traditional techniques suffer of some limitations: long formation times (24-48 h), traces of the organic solvents used to prepare the polymer solution, closed structures or collapse of the porous morphology during solvent extraction [8].
Drying assisted by supercritical CO_2 (SC-CO_2) has been proposed as alternative to traditional processes and it has been demonstrated to be an efficient technique to obtain aerogels that maintain their native morphology at micro and nanoscale [4, 8]. This result is possible since this process is

carried out at negligible surface tension and the supercritical mixture solvent+CO_2 shows a large mass transfer coefficient. These characteristics avoid structure collapse and the solvent is rapidly extracted from the polymeric matrix [9].
Valentin et al. [10] produced by supercritical drying, alginate gels characterized by an high surface area (>300 m^2/g). Robitzer et al. [11] used the same technique to prepare Ca-alginate aerogels that showed a nanofibrous morphology with a mean fiber size of about 8 nm. Veronovski et al. [12] produced aerogels of natural polysaccharides loaded with nicotinic acid, via sol-gel and supercritical drying. The results indicated that using the internal setting cross-linking method for obtaining monolithic aerogels, nicotinic acid was released in a more controlled manner. However, with increasing alginate concentration, more compact and cross-linked aerogels were formed.
Rui Rodrigues et al. [13] prepared alginate gels in the presence of divalent cations, such as calcium and copper. These authors studied cooper ions binding during Cu-alginate gelation, obtaining quantitative information about the amount and kinetics of cation binding. The results indicated that copper binding during gelation occurred until a Langmuir-type equilibrium was reached between bound and free ions in the gel-contacting solution. Goh et al. [14] investigated the antimicrobial activities of calcium ions and other cross-linking agents of alginate dressings, as well as their compatibility with commonly used topical antimicrobials. They found that calcium ions exhibited very weak antimicrobial activity and higher fractional inhibitory concentration than the other cross-linking agents. On the other hand, aluminium, zinc and copper ions exhibited higher antimicrobial activities, but insignificant interactions with the antimicrobials. Erol et al. [15] synthesized and characterized new boron-containing bioactive glass-based scaffolds coated with alginate cross-linked with copper ions. Scanning electron microscopy indicated that the alginate effectively attached on the surface of the scaffolds leading to a homogeneous coating. This coating improved the scaffold bioactivity and mechanical properties; whereas, copper release studies showed that the alginate-coated scaffolds allowed controlled release of copper ions. Klinkajon et al. [16] incorporated copper (II), with antimicrobial activity, into an alginate dressing to minimize infection in a wound. In the first step, solid alginate films were prepared using a solvent-casting method from soft gels of alginate solutions that had been lightly cross-linked using a copper (II) sulfate solution. In the second step, the films were further cross-linked in a corresponding Cu^{2+} sulfate solution using a dipping method to improve their stability. Alginate solution at 2% w/v and Cu^{2+} sulfate solution at 2% w/v in acetate buffer at low pH, provided soft films with swelling behavior. An increase in either Cu^{2+} ion concentration or cross-linking time, led to hydrogels with more densely-cross-linked networks that limited water absorption. These hydrogels showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* and *Streptococcus pyogenes*.
Therefore, the aim of this work is to produce alginate aerogels characterized by a cylindrical shape with an annular internal section, by supercritical drying for vascular applications. These alginate aerogels were crosslinked with calcium or copper ions to determine differences in terms of morphology e chemical properties in the obtained samples. Therefore, these structures were characterized by scanning electron microscopy, BET specific surface and by UV/Vis spectrophotometry to measure Cu free residues that should be useful for vessels formation.

MATERIALS AND METHODS

Sodium alginate, calcium chloride (anhydrous powder) and copper sulfate (II) (anhydrous, powder, ≥99.99% trace metals basis) were bought from Sigma Aldrich. CO_2 (purity 99.9%) was supplied by Morlando Group S.R.L. (Sant'Antimo, NA - Italy).
Alginate solutions were prepared in water at different polymer concentrations. They were mixed at room temperature, using a magnetic stirring at 200 rpm for 24 h. Samples of desired shape were obtained using an extruder, shown in Figure 1. This extruder consisted of an AISI 316 vessel with an internal diameter of 19 mm. Compressed air at 1 bar, pushed the polymer solution through a Teflon disk, placed above a 33 mm long pin, fixed on a plate of the same diameter as the Teflon disk. When

the sample came out of the extruder, it immediately was in contact with the coagulation bath, to stabilize its shape. In this way, a cylindrical hydrogel with an annular internal section was obtained.

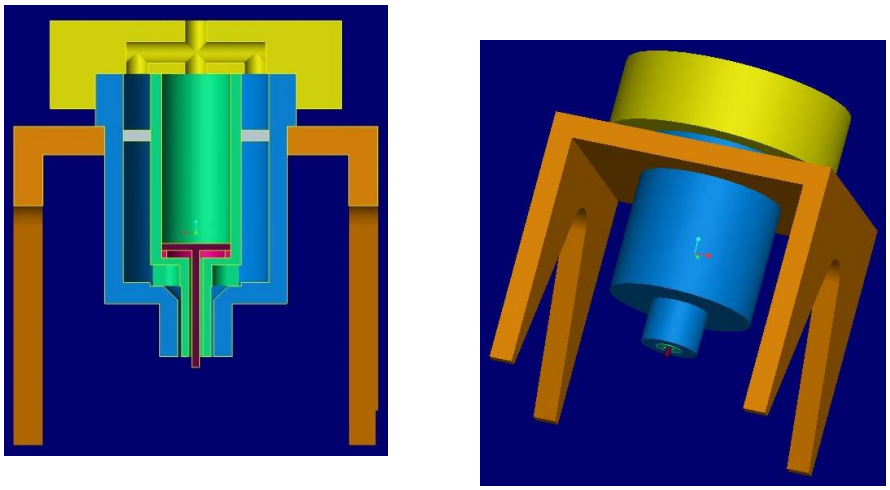


Figure 1. Representation of the extruder used to obtain cylindrical alginate aerogels with an annular internal section.

After that, alginate sample was immersed in water/ethanol baths at increasing ethanol percentage by volume (10, 30, 50, 70, 90, 100% v/v). Each step lasted 2 h, except for the last one of 24 h. Water substitution with ethanol was performed since it is known from the literature that CO_2 and water have low chemical affinity [9]; therefore, this step was required to correctly perform the supercritical drying process.

Alginate aerogels were obtained using a laboratory plant that consisted of a 316 stainless steel cylindrical high-pressure vessel with an internal volume of 200 mL, equipped with a high pressure pump (mod. LDB1, Lewa, Germany) used to deliver liquid CO_2. Pressure in the vessel was measured by a test gauge (mod. MP1, OMET, Italy) and regulated using a micrometering valve (mod. 1335G4Y, Hoke, SC, USA). Temperature was regulated using PID controllers (mod. 305, Watlow, USA). At the exit of the vessel, a rotameter (mod. D6, ASA, Italy) was used to measure CO_2 flow rate. The vessel was filled with SC-CO_2; when the required pressure and temperature were obtained (200 bar and 45 °C), drying was performed using a SC-CO_2 flow rate of about 1 kg/h for 4 h. A depressurization time of about 30 min was used to bring back the system at atmospheric pressure.

Field Emission Scanning Electron Microscopy (FE-SEM) was performed on alginate aerogels previously 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 120 s and analyzed using a FESEM (mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany) to determine the aerogels morphology and to measure the mean diameter of the nanofibers forming the structure.

BET specific surface area was determined by Nitrogen sorption using a Nova 1200e Surface Area & Pore Size Analyzer (Quantachrome Instruments, Florida, USA). About 0.2 g of sample were first degassed at 60 °C for 2 h and, thus, analyzed by N_2 adsorption at -196 °C.

Free Cu residues released from alginate aerogels, were measured in continuous using a Varian (mod. Cary 50) UV/Vis spectrophotometer, reading the absorbance of the sample at 808 nm (the wavelength at which Cu shows maximum absorption) at room temperature. During the analysis, the alginate aerogel was immersed in a Phosphate Buffer Solution (PBS) at pH=7.4.

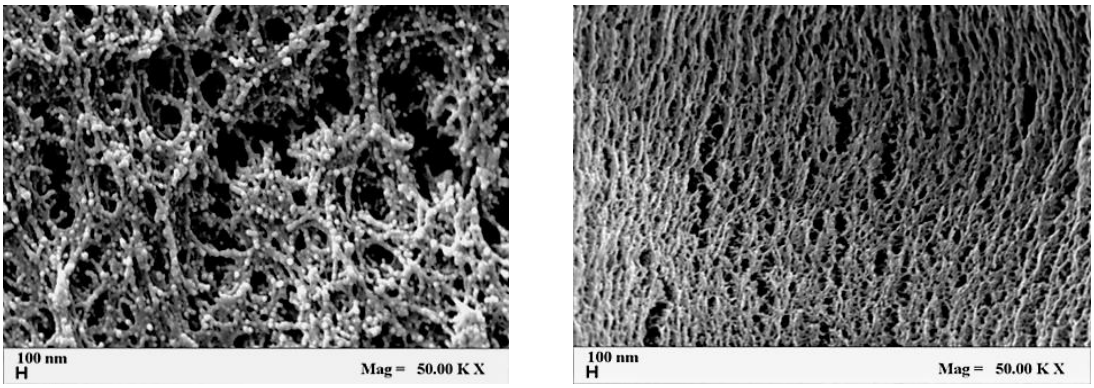
RESULTS AND DISCUSSION

Alginate solutions in water were prepared at different polymer concentrations (5, 10 and 15% w/w). Then, they were extruded using the extruder described in the previous section to obtain a cylindrical sample with an annular internal section. In order to stabilize this shape, the extruded sample was directly immersed in a coagulation bath of $CaCl_2$ at 5% w/w or $CuSO_4$ at 1.5% w/v for 24 h and at room temperature.

The Ca-Alg-hydrogels or Cu-Alg-hydrogels, were processed by supercritical drying at 200 bar and 45 °C, after water substitution with ethanol, as described in Materials and Methods.
In Figure 2, an example of a Ca-Alg-aerogel is shown. Therefore, the cylindrical annular shape was maintained also after supercritical processing.

Figure 2. Example of a Ca-Alg-aerogel.

From a microscopic point of view, a nanofibrous and porous morphology was observed both for Ca-Alg-aerogels and Cu-Alg-aerogels. In particular, SEM images reported in Figure 3a-b are related to Ca-Alg-aerogel and Cu-Alg-aerogel at 5% w/w; the same morphologies were observed for the other polymer concentrations. Ca-Alg-aerogel was characterized by a nanofibous morphology with a mean fibers diameter of about 200 nm. Polymer nanoparticles precipitated on these nanofibers were also observed. Cu-Alg-aerogel showed a regular nanofibrous morphology and nanopores; in this case, the mean fibers diameter was of about 100 nm. These results confirm that supercritical processing avoided gel collapse during the experimentation, since a supercritical mixture between CO_2 and ethanol was formed with a negligible surface tension at these operative conditions [9].



(a) **(b)**

Figure 3. SEM images of (a) 5% w/w Ca-Alg-aerogel and (b) 5% w/w Cu-Alg-aerogel.

Even if in both cases a nanofibrous morphology was obtained, different specific area was measured by BET analysis. In Table 1, specific area values for all the samples produced in this work are reported. In particular, a larger specific area was measured for Ca-Alg-aerogels with respect to the Cu-Alg-aerogels, that ranged between about 297 and 200 m^2/g; whereas, for Cu-Alg-aerogels, it ranged between about 111 and 19 m^2/g. These results can be explained considering that the Cu-Alg-aerogels were characterized by a more compact structure with respect to Ca-Alg-aerogels at the same polymer concentration, as it is also possible to observe from SEM pictures in Figure 3a-b; this difference was more relevant when the alginate concentration increased in the starting sample. This morphological behavior is also a consequence of the chemical mechanism during the crosslinking step. In the literature is reported that alginate can be crosslinked using bivalent cations, such as Cu, Ca, Ba, etc.; but, the chemical affinity between alginate and these ions increases in the following order: Pb> Cu> Cd> Ba> Sr> Ca>Co [17]. Therefore, chemical affinity between Ca and alginate is lower with respect to the chemical affinity between Cu and alginate. As a consequence, the reaction

velocity is higher when $CuSO_4$ was used as crosslinking agent, producing also a more compact structure characterized by a lower specific area.

Sample	Specific area, m^2/g
Ca-Alg 5% w/w	296.5
Ca-Alg 10% w/w	219.4
Ca-Alg 15% w/w	201.0
Cu-Alg 5% w/w	111.0
Cu-Alg 10% w/w	25.20
Cu-Alg 15% w/w	19.20

Table 1. BET results of the produced alginate aerogels.

In the last part of this work, free Cu residues in the Cu-Alg-aerogels were measured using the procedure described in Materials and Methods section. Cu-Alg-aerogel was immersed in PBS at pH of 7.4 to simulate the body environment. In Figure 4, the Cu release curves versus time are reported. The measured maximum Cu concentration was about 160 ng for 5% w/w Cu-Alg-aerogel. This result is a consequence of the lower polymer concentration in the sample at a fixed sample volume and crosslinking amount; i.e., using the same aerogel volume and $CuSO_4$ concentration in the coagulation bath, the polymer chains reacting with Cu were lower. Therefore, a larger quantity of free Cu residues from the alginate aerogel at lower polymer concentration was expected. The useful released Cu amount that can induce angiogenic activity is comprised between 22 and 220 ng [18]: therefore, also the 15% w/w Cu-Alg-aerogel, that released about 98 ng, was useful for this biomedical application.

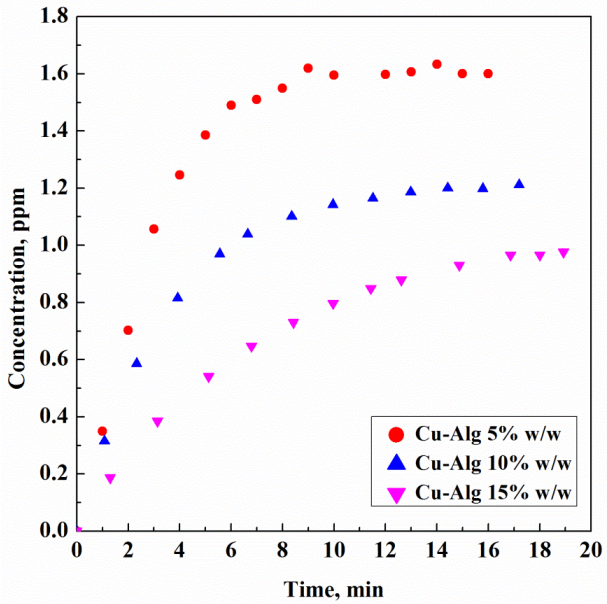


Figure 4. Diagram representing Cu concentration released during the time from Cu-Alg-aerogels.

CONCLUSIONS AND PERSPECTIVES

In this work, Ca-Alg-aerogels and Cu-Alg-aerogels were successful produced by supercritical drying. The nanofibrous morphology of both systems confirmed that supercritical processing preserved this delicate structure also at nanometric scale. Moreover, in the case of Cu-Alg-aerogels, the Cu amount released from the samples was suitable for angiogenic function in the host since it was larger than 22 ng. These chemico-physical properties, coupled to the proper macroscopic annular cylindrical shape, make these alginate aerogels suitable for potential vascular applications.

In the next future, these alginate aerogels could be tested with cells to study their behavior in dependence on the kind of crosslinking agent used; also mechanical properties could be measured

and optimized to confer to these scaffolds the proper mechanical resistance and elasticity typical of a blood vessel.

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