Complete Glutaraldehyde elimination during Chitosan hydrogel drying by SC-CO_2 processing

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Abstract

Chitosan (CH) is one of the polymers most frequently proposed in form of hydrogels for tissue engineering (TE) applications. It is often crosslinked with the aim of improving its stability and mechanical properties. In this work, for the first time, a supercritical carbon dioxide (SC-CO_2) gel drying process was proposed to obtain CH scaffolds and simultaneously eliminate unreacted glutaraldehyde (GTA), taking advantage of its solubility in supercritical mixtures.

SC-CO_2 gel dried crosslinked CH aerogels showed a nanofibrous structure characterized by an average diameter of about 100 nm. No collapse of the nanostructure was observed in the aerogels, due to the peculiarities of supercritical fluids (near zero surface tension). Moreover, crosslinked CH showed a thermally stable structure, as determined by TGA analysis. The hypothesis that a supercritical mixture is able to efficiently extract GTA residues entrapped in the gel matrix was also verified: negligible residues of GTA were found at the end of release experiments; in particular, at the best operating conditions, a GTA concentration equal to 0.013 ppm was detected. Therefore, the produced crosslinked CH aerogels can be effectively and safely used for TE applications.

Keywords: Glutaraldehyde, Chitosan, Supercritical CO_2, Aerogel, Scaffold.

1 Introduction

Tissue damage or organ failure are among the most frequent and devastating problems in medicine. Current therapeutic approaches (allografts, xenografts, autografts and implantation of biomedical devices) have largely improved the quality of life; but they are associated with some limitations including donor availability, infection transmission, poor integration and potential rejection of the implant. Tissue engineering (TE) has the aim to overcome such limitations finding new therapies for the treatment of tissue diseases, with the basic idea to repair or re-create tissues or organs, restoring impaired functions (Barry, Gidda, Scotchford & Howdle, 2004; Eichhorn & Sampson, 2005).

Several TE strategies depend on the preparation of polymeric scaffolds, that can operate as a synthetic extra cellular matrix (ECM) to organize cells into a three-dimensional architecture and to stimulate formation and growth of a desired tissue (Yang, Leong, Du & Chua, 2001). Depending on the tissue of interest and on the specific application, the required scaffold material and its properties can be quite different; but, for all tissues, it is required (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) nanostructural characteristics mimicking ECM; 4) mechanical properties suitable to maintain the predesigned tissue structure and to support the specific loadings applied to the original tissue; 5) biocompatibility and a proper degradation rate; 6) absence/reduced toxic and inflammatory response.

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

Among the polymeric materials proposed for TE applications, water-soluble polymers have been frequently used in form of hydrogels (Park & Lakes, 2007). They are formed by hydrophilic

polymer chains and can be either of synthetic or of natural origin. The structural resistance of hydrogels depends on crosslinks formed between polymer chains via chemical bonds and/or physical interactions. Physically bonded hydrogels used in TE applications have structural properties similar to the ECM, can be processed under moderately mild conditions, , and can be used in a minimally invasive manner (Lee & Mooney, 2001). However, it is difficult to accurately control gel pore size, chemical functionalization, dissolution and degradation; moreover, mechanical properties are limited. Consequently, these hydrogels often provide inconsistent in vivo performances (Dash, Chiellini, Ottenbrite & Chiellini, 2011). A way to improve hydrogel properties is the creation of irreversible networks formed by chemical bonds. In these cases, polymer chains are covalently bonded together by small cross-linker molecules, secondary polymerization, or irradiation.

Hydrogels can also be used or stored in dried form; the most used technique to obtain these structures is freeze-drying. This technique is suitable for a wide range of hydrogel materials, including natural (Jin, Moreira Teixeira, Dijkstra, Karperien, Zhong & Feijen, 2008; Lv, Hu, Feng & Cui, 2008) and synthetic hydrophilic polymers (Lee, Kang, Kim & Son, 2008; Lin, Yu & Yang, 2006). However, hydrogels architecture is extremely sensitive to the quenching process and can totally or partially collapse due to the interfacial tension of the liquid during solvent evaporation (Ho et al., 2004). Another limitation of the freeze-drying process is due to the frequent formation of a surface skin and to the partial collapse of the structure at the scaffold-air interface; in addition, freeze-drying is energy intensive and requires relatively long processing times for the solvent removal (Quirk, France, Shakesheff & Howdle, 2004).

Chitosan (CH) is obtained by deacetylation of the natural biopolymer chitin. Both chitosan and chitin are chemically similar to cellulose, differing only by the functional group situated at Carbon-2 of the monomeric unit. The presence of free amine groups in chitosan enhances the solubility and reactivity of this polymer with respect to chitin and cellulose. Some products obtained by chemical modification of chitosan found applications in various fields (Dash, Chiellini,

Ottenbrite & Chiellini, 2011; Dutta, Dutta & Tripathi, 2004; Harish Prashanth & Tharanathan, 2007; Ravi Kumar, 2000). For example, covalent chitosan cross-linked hydrogels are obtained by attacking photo-reactive or enzyme-sensitive molecules on the polymer, followed by their exposure to UV or sensitive enzymes. The properties of cross-linked hydrogels mainly depend on their cross-linking density and by the ratio between cross-link molecules and polymer repeating units (Park, Park & Shalaby, 2011).

Chitosan hydrogels are frequently crosslinked using glutaraldehyde (GTA) that is one of the most effective crosslinking agents; the interest in modifying chitosan using GTA has recently increased in many potential applications, especially in biomedical and environmental field (Alves & Mano, 2008; Berger, Reist, Mayer, Felt, Peppas & Gurny, 2004; Dash, Chiellini, Ottenbrite & Chiellini, 2011; Krajewska, Zaborska & Leszko, 1997; Kurita, Yoshino, Nishimura, Ishii, Mori & Nishiyama, 1997; Ma et al., 2003; Payne, Chaubal & Barbari, 1996). The limitations due to the use of GTA (frequently used as a powerful sterilizing agent) are related to its high cytotoxicity (Zeiger, Gollapudi & Spencer, 2005). This characteristic is known by a long time; Speer et al. found that even a 3.0 ppm GTA concentration inhibited fibroblast growth in tissue culture at 99% (Speer, Chvapil, Eskelson & Ulreich, 1980). Therefore, it is desirable to efficiently eliminate GTA after crosslinking (Choy, Cheng, Choi & Kim, 2008); unfortunately, this operation is usually not performed, and/or its success is limited by mass transfer resistance encountered by liquid solvents in solid media.

Supercritical carbon dioxide (SC-CO_2) has been successfully used in several improved processes to produce micro and nanoparticles (Adami, Liparoti & Reverchon, 2011; De Marco, Knauer, Cice, Braeuer & Reverchon, 2012; Du, Shen, Tang, Guan, Yao & Zhu, 2014; Liparoti, Adami & Reverchon, 2012; Marra, De Marco & Reverchon, 2012; Montes, Gordillo, Pereyra, De los Santos & Martínez de la Ossa, 2014) and porous materials (Baldino, Cardea, De Marco & Reverchon, 2014; Baldino, Cardea & Reverchon, 2014; Cardea, Baldino, Pisanti & Reverchon, 2014; Cardea, Baldino, Scognamiglio & Reverchon, 2014; Cardea & Reverchon, 2011; Duarte,

Mano & Reis, 2009; Karimi, Heuchel, Weigel, Schossig, Hofmann & Lendlein, 2012). Recently, a SC-CO_2 based gel drying process has also been proposed to produce 3-D scaffolds (Pisanti, Yeatts, Cardea, Fisher & Reverchon, 2012; Reverchon, Cardea & Rapuano, 2008), preserving macro and nanostructure of the gel. Indeed, efficient solvent elimination and short processing times can be obtained due to the large affinity of SC-CO_2 with almost all the organic solvents. In this specific process, SC-CO_2 forms a supercritical mixture with the organic solvent used to produce the gel, that is, then, eliminated maintaining zero surface tension inside its solid structure. This operation avoids gel collapse. However, SC-CO_2 shows a very limited affinity with water at the ordinary temperatures and pressures used in SC-CO_2 processing; for example, at 40 °C and 100 bar, water solubility is around 0.5 % w/w (Sabirzyanov, Il'in, Akhunov & Gumerov, 2002). Therefore, the described process is not directly applicable to polymeric hydrogels, and some water-ethanol exchange steps have to be performed to substitute with ethanol the water contained in the gel before SC-drying (Della Porta, Del Gaudio, De Cicco, Aquino & Reverchon, 2013; García-González, Alnaief & Smirnova, 2011).

In this work, we propose, for the first time, SC-CO_2 gel drying of CH scaffolds with the simultaneous elimination of unreacted GTA, taking advantage of GTA solubility in supercritical mixtures. Operating in this manner, we can obtain improved CH scaffolds for TE applications that maintain the original nanofibrous structure of the gel, and do not contain residues of the highly cytotoxic crosslinking agent that, if not removed, does not allow the proliferation of cells in the scaffold. CH-GTA aerogels will be characterized from a macroscopic and microscopic point of view and a study of the release of GTA residues will be performed.

2 Materials, apparatuses and methods

Chitosan with Low M_W (Deacetylation 75 – 85 %, viscosity 20 - 300 cps) and Medium M_W (Deacetylation 75 – 85 %, viscosity 200 - 800 cps) were purchased from Sigma-Aldrich (St. Louis, MO); Acetic Acid glacial (99.9 % purity) and Sodium Hydroxide (NaOH) were bought from Carlo

Erba Reagenti (Rodano, Mi - Italy); Carbon Dioxide (99 % purity) was bought from SON (Società Ossigeno Napoli - Italy). Glutaraldehyde solution 25 % w/w in water was purchased from Sigma-Aldrich. Distilled water was produced using a laboratory water distiller supplied by ISECO S.P.A. (St. Marcel, Ao - Italy). All materials were used as received.

2.1 Preparation of Chitosan aerogel

Two kinds of CH (Low M_W and Medium M_W) were used, starting from two different CH concentrations (2 and 5 % w/w) in acid water (water to acetic acid ratio 95:5). The best preparation of CH hydrogel was the following: Chitosan Medium M_W (0.40 g) was dissolved in 20 mL of water/acetic acid solution (pH 2.43) to obtain a CH concentration of 2 % w/w. NaOH (1 M in water, pH 14) was slowly added to this solution and the changes in pH were registered, each added mL, by a pH-meter. At the same time, the gel formation was monitored, until the attainment of the maximum gel point that was reached at pH 12.5. Then, the gel sample was repeatedly washed with distilled water to remove the excess of NaOH solution.

CH hydrogels were crosslinked using a GTA solution 0.25 % w/w in water. The crosslinking solution was obtained by diluting 1 mL of GTA (25 % w/w) in 99 mL of water. Different ratios CH gel/GTA were used (see Table 1), to identify the best operating conditions: CH:GTA 32:1, 16:1, 8:1, 4:1 and 1:1; GTA content was increased to the maximum ratio (1:1) to verify the efficacy of the SC-CO_2 purification process on crosslinked CH aerogel.

CH:GTA	**32:1**	**16:1**	**8:1**	**4:1**	**1:1**
CH hydrogel, mL	10	3	3	3	3
GTA 0.25 % w/w, mL	0.3	0.2	0.4	0.75	3

Table 1. CH and GTA amounts used for the crosslinking reaction.

The crosslinking reaction was performed at room temperature, adding GTA solution to CH gel, under Vortex shaking for 1 min. The system was left for three days in quiet. Then, the crosslinked sample was repeatedly washed with distilled water. SC-CO_2 drying cannot be directly applied in the case of hydrogels, since SC-CO_2 shows a very reduced affinity with water (Diamond & Akinfiev, 2003). Therefore, before drying, the samples were immersed in an ethanol bath for 24 h at room temperature, to allow the complete replacement of water with ethanol inside the samples.

2.2 SC-CO_2 gel drying description

Samples were prepared in a homemade 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 (mod. LDB1, Lewa, Germany) used to deliver SC-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 along the plant was regulated using PID controllers (mod. 305, Watlow, Italy). At the exit of the vessel, a rotameter (mod. D6, ASA, Italy) was used to measure CO_2 flow rate.

SC-CO_2 gel drying was performed 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; drying lasted 8 h. A depressurization time of 20 min was used to bring back the system at atmospheric pressure.

2.3 Analytical methods

To perform Field Emission Scanning Electron Microscopy (FESEM), the samples 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 analyzed using a FESEM (mod. LEO

1525, Carl Zeiss SMT AG, Oberkochen, Germany) to evidence the micro and nanostructure and to measure the diameter of the fibers forming the structure.

A Perkin Elmer Lambda 900 Spectrophotometer (Milano, Italy) was used, working in time-drive mode at a fixed wavelength of 234 nm (maximum absorption for GTA) for the study of GTA release from the CH aerogels. The concentration of GTA released in the Phosphate Buffer Solution (PBS) was detected by UV-absorption spectra recorded as a function of time. In a typical experiment, 15 mL of CH hydrogel were immersed in 3 mL of PBS buffer (pH 7.4) and glycine 0.1 M (PBS:Gly = 0.43). Glycine role is to suppress the pH decrease normally observed in sodium phosphate buffer salt, possibly by reducing the nucleation rate of salt and thereby decreasing the extent of buffer salt crystallization. GTA absorbance was measured at 234 nm, using a UV-Vis spectrophotometer at room temperature (Bigi, Cojazzi, Panzavolta, Rubini & Roveri, 2001).

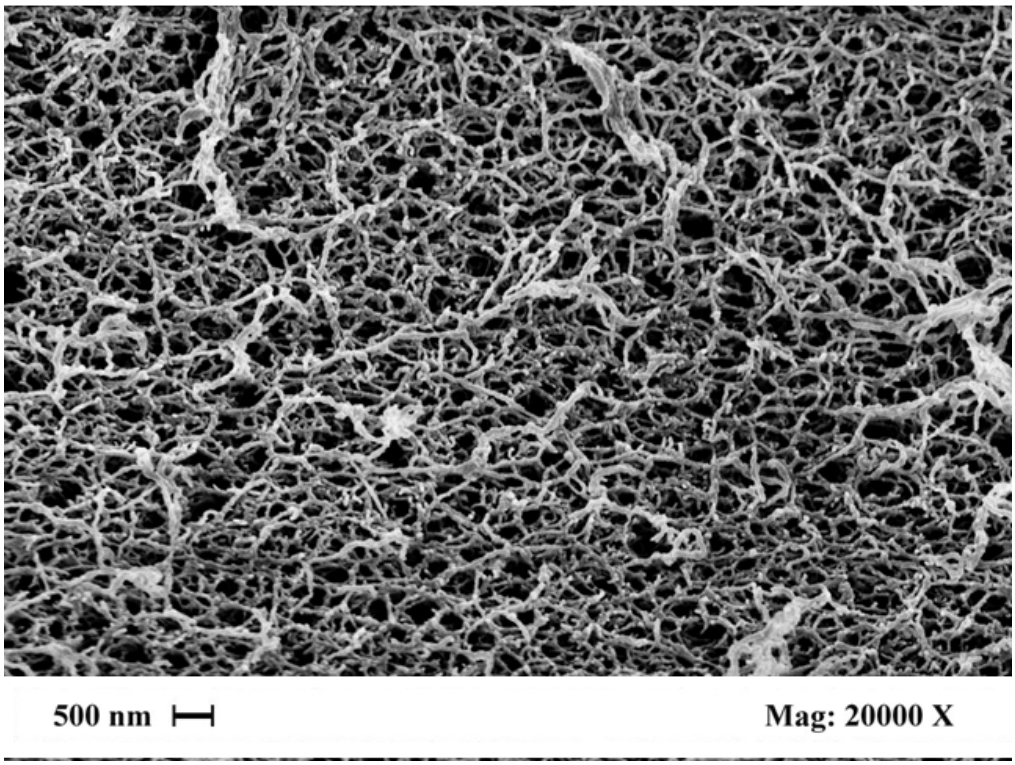
FT-IR spectra were recorded using a Thermo Nicolet Nexus Spectrometer (Rodano (MI), Italy), equipped with Attenuated Total Reflection (ATR) sampling, working with a Zinc selenide crystal.

The thermal behavior of CH 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.

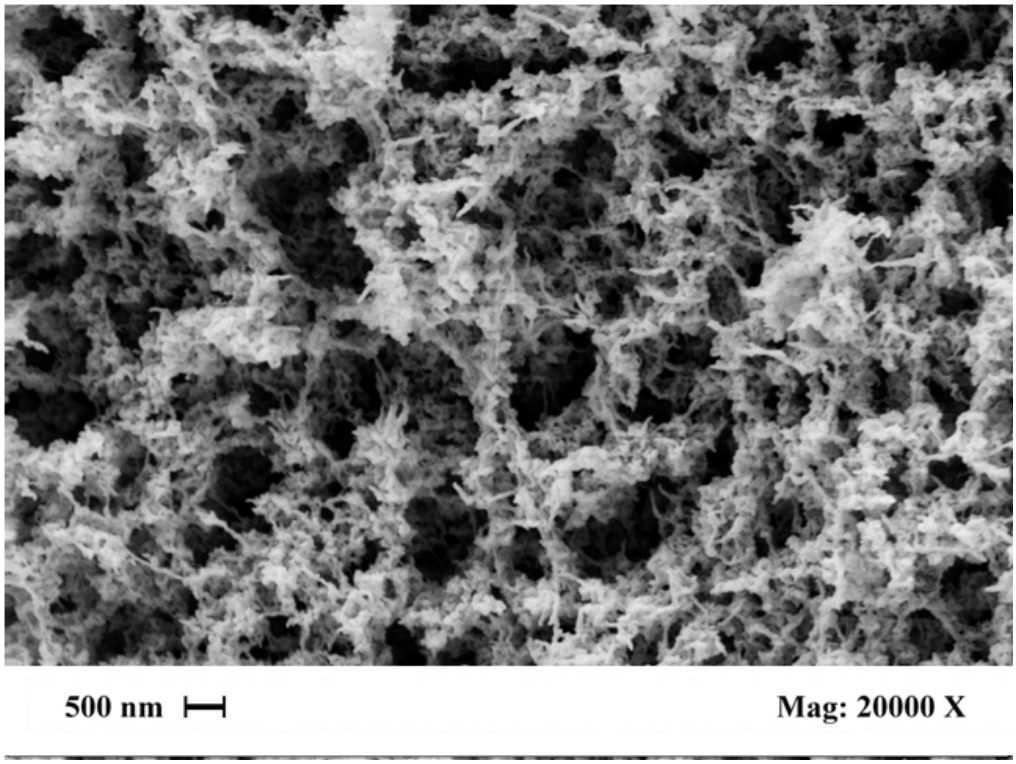
3 Results and Discussion

In the first part of the study, some experiments were performed to optimize the CH hydrogel in water. We found that, at the end of the drying process, the samples maintained the same shape and volume, which is the first evidence that the delicate nanofibrous architecture of CH gels was not destroyed. This evidence was confirmed by the FESEM images, taken at the same enlargement, reported in Figure 1a-d, in which both CH aerogel (Figure 1a) and crosslinked CH aerogel (Figure 1b-d) showed the same nanofibrous morphology. The nanofibers are very regular and uniformly distributed along the samples section and have an average size of about 100 nm. The only

difference between Figure 1 images is related to the different arrangement of the nanofibers that could depend on the quantity of GTA used for crosslinking.



(a)



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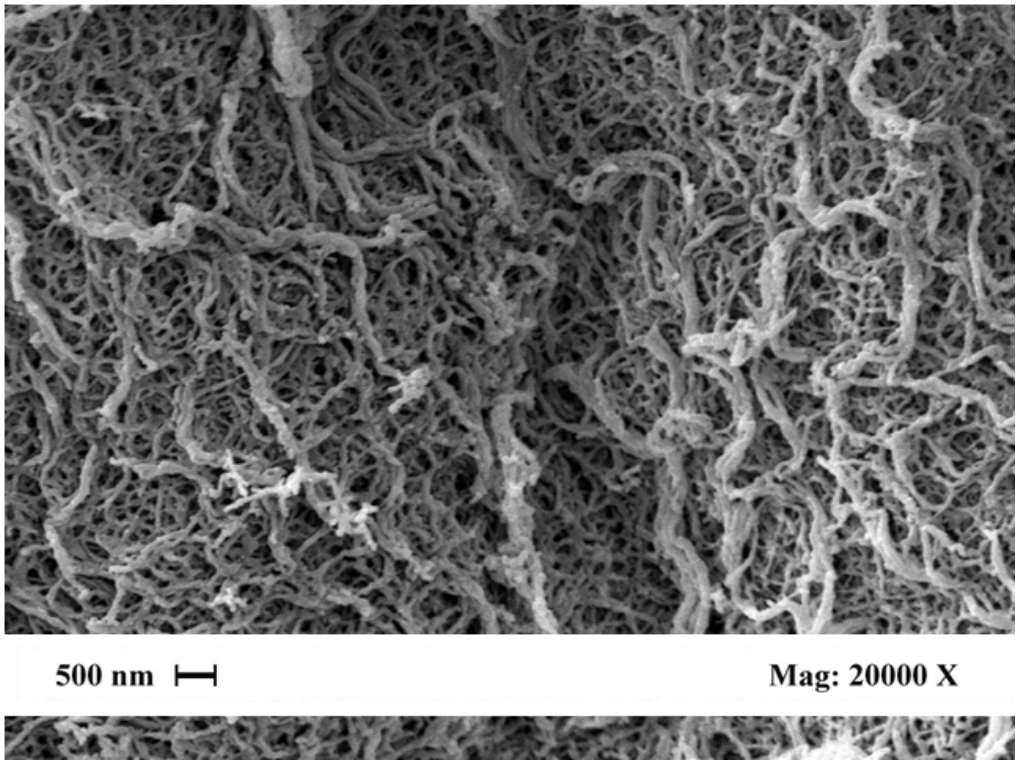

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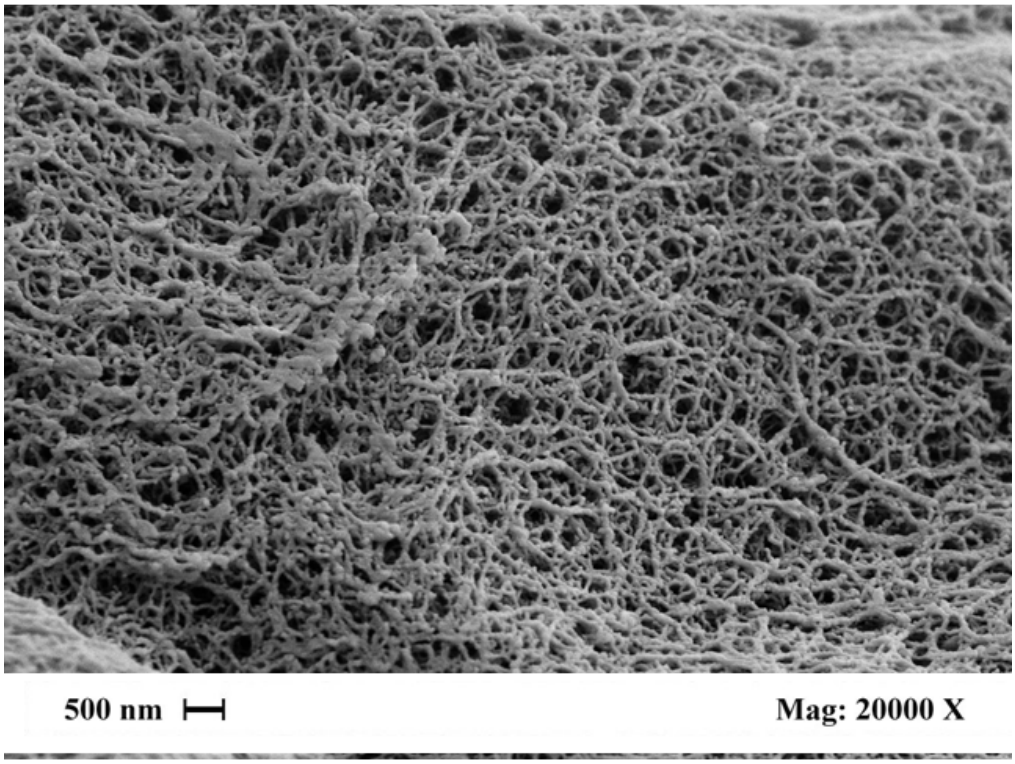

(d)

Figure 1. FESEM images of Chitosan medium M_w (2 % w/w): (a) CH aerogel section; (b) CH:GTA 16:1 aerogel section; (c) CH:GTA 8:1 aerogel section; (d) CH:GTA 4:1 aerogel section.

Also the samples surface maintained the uniform and nanofibrous morphology, as shown, for example, in Figure 2, where a FESEM image of the surface of a CH:GTA 4:1 aerogel is reported. On the contrary, as discussed in the Introduction, aerogels produced by freeze-drying show closed surfaces, due to the force exerted by the surface tension during the solvent extraction from the polymeric matrix, which causes the polymer collapse.

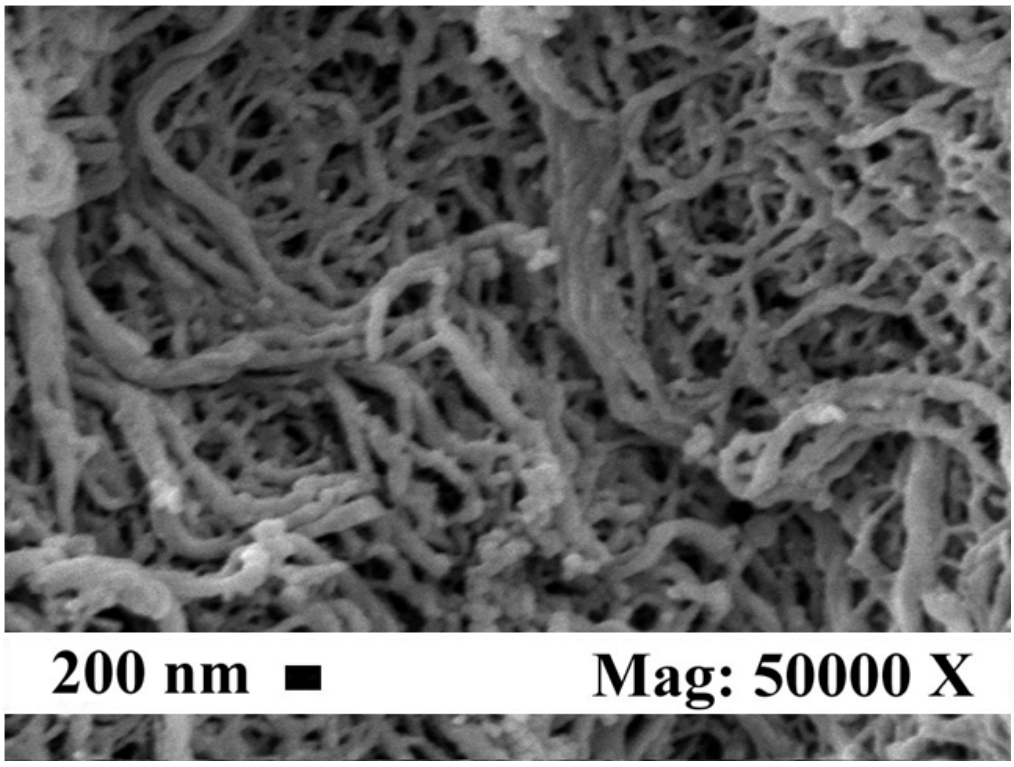

Figure 2. FESEM image of CH:GTA 4:1 aerogel surface.

This result is important to favor the interaction of cells with the aerogel, since open skins are required for the cell inoculation, adhesion and proliferation; it means that this morphology improves the efficiency of the cells culture. Summarizing, nanostructured gel morphology was preserved in all the cases and it is similar to that of the ECM proteins of the native tissues, that show an average fibers diameter between 50-500 nm; i.e., 1 to 2 orders of magnitude smaller than the living cells. This characteristic allows the cells to be in direct contact with many ECM fibers, thereby defining their three dimensional orientation (Barnes, Sell, Boland, Simpson & Bowlin, 2007).

3.1 Glutaraldehyde release from Chitosan aerogel

The second problem in CH hydrogel drying is the entrapment inside the structure of unreacted GTA. It can be reduced by washing several times the samples with ethanol to extract at least part of the GTA and using calibrated quantities of GTA to induce crosslinking, reducing at a minimum the unreacted material. However, since GTA (as shown in the Introduction) is a powerful sterilizing agent, the possibility of extracting its residues during the drying process can generate a great improvement of the final product characteristics, in view of its application for cells cultivation. Indeed, being GTA a small molecule, its solubility in SC-CO_2 (not known at the best of our knowledge) could be low. Moreover, during SC-drying, CO_2 extracts ethanol contained in the alcogel, forming a SC-mixture ethanol-CO_2. This mixture shows more affinity with GTA than SC-CO_2 alone, due to the presence of ethanol in which GTA is largely soluble. In addition, gas-like diffusivity of the supercritical ethanol-CO_2 mixture can allow a more efficient GTA extraction with respect to the one attainable using liquid ethanol. To verify experimentally this hypothesis, a series of GTA release experiments was performed. GTA concentration was measured using a calibration curve obtained using known quantities of GTA; the released GTA curves and the final concentration values (in terms of ppm) are respectively reported in Figure 3 and Table 2.

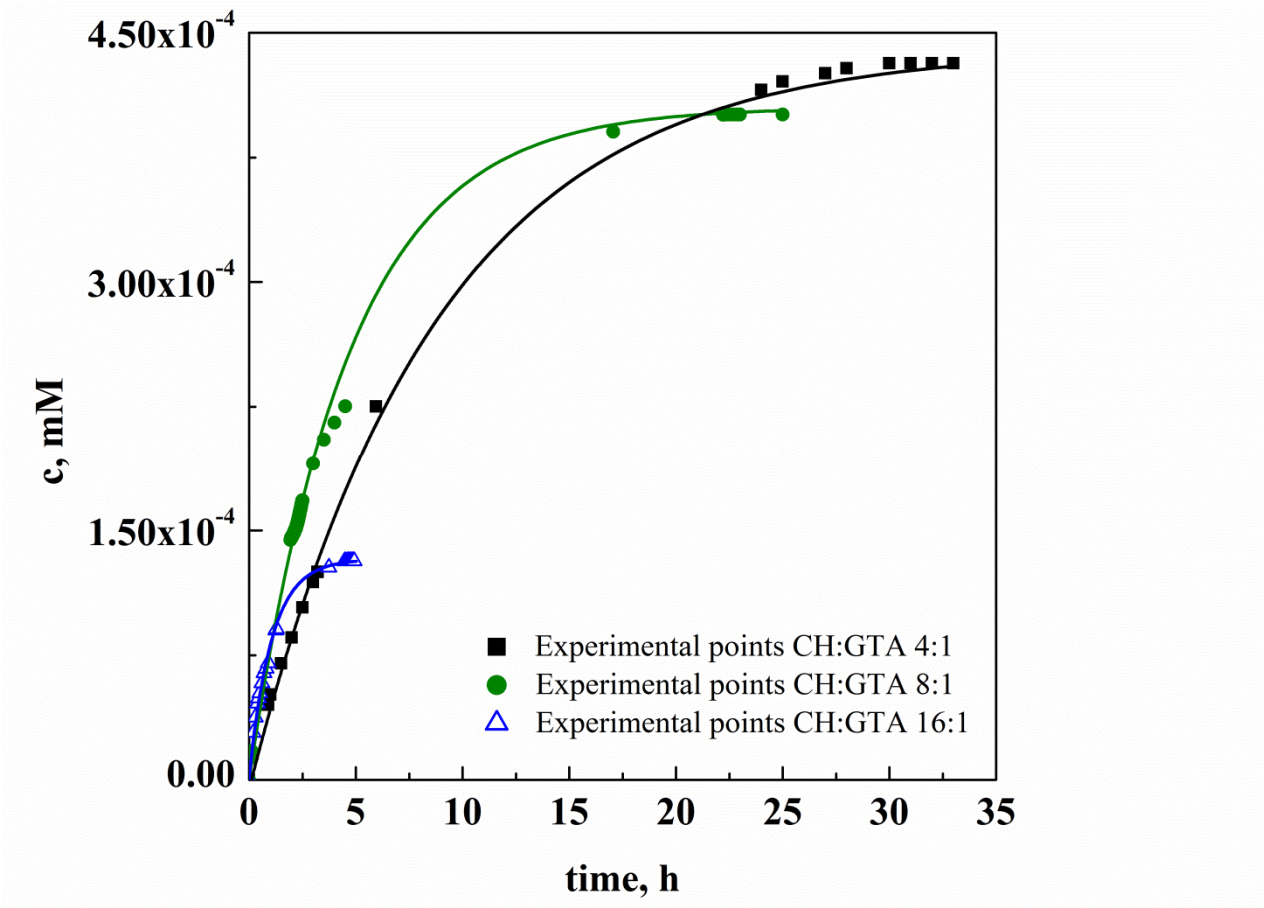

Figure 3. GTA release from CH aerogels at 2 % w/w.

Aerogel	GTA concentration, ppm
CH:GTA 1:1	77.7
CH:GTA 4:1	$4.32 \cdot 10^{-2}$
CH:GTA 8:1	$4.01 \cdot 10^{-2}$
CH:GTA 16:1	$1.32 \cdot 10^{-2}$

Table 2. Maximum GTA concentration measured in the release medium.

The sample CH:GTA 1:1, in which the quantity of GTA used for crosslinking is in a large excess, was not reported in Figure 3. In all the other cases, the obtained asymptotic curves were fitted using an exponential equation. The GTA released from the samples is maximum (about 0.04 ppm) for the CH:GTA ratios equal to 4:1 and 8:1 and is about 0.01 ppm for CH:GTA ratio equal to

16:1. These amounts are, for all the samples, negligible if compared to the value of 3 ppm reported in literature as the GTA residue that blocks, almost completely, cells reproduction (Speer, Chvapil, Eskelson & Ulreich, 1980). These values clearly indicate that these materials can be readily used for tissue engineering applications.

3.2 FTIR analysis

IR spectroscopy was used to evaluate the structural modifications induced by the crosslinking of chitosan with GTA. In Figure 4, FTIR spectra obtained with ATR method on samples of CH 2 % w/w are shown. In particular, the following sample curves are reported: pure CH (used as a reference), CH:GTA 16:1, 8:1, 4:1 and 1:1. The last sample (1:1) contained a large GTA excess and was used only to verify the efficiency of the SC-CO_2 based purification process on crosslinked aerogels.

FTIR spectrum of pure CH (Figure 4, first curve from the top) shows the typical band at 3360 cm^{-1} of OH and NH_2 stretching. The peak at 2874 cm^{-1} belongs to stretching vibration of CH groups. In all the spectra, two peaks are visible at 1650 cm^{-1} and 1640 cm^{-1}, which can be attributed to C=O stretching and NH bending of amide groups (note that CH we used had a deacetylation degree of 80 %). The peak at 1376 cm^{-1} can be assigned to methyl groups of the polymer and the one at 1420 cm^{-1} to the bending of O–H and C–H groups.

For all the samples (from 16:1 to 1:1 ratios), a peak at 1590 cm^{-1}, belonging to the imine C=N bond, formed between the amine residues of CH and the aldehyde terminals of the GTA, is well detectable. At the same time, it can be noticed that only in the CH:GTA 1:1 curve, a small peak at 1750 cm^{-1}, typical of C=O carbonyl group, is slightly visible. This peak indicates a non-negligible presence of GTA in the sample 1:1. The large peaks detected at around 1070 e 1024 cm^{-1} correspond to the CO bending vibration, typical of CH saccharidic structure.

Therefore, from FTIR analysis, two considerations can be made: 1) the cross-linking process of CH with GTA has been successfully obtained, as demonstrated by the presence of the well

detectable peak at 1590 cm^{-1} in all the curves of cross-linked samples (imine C=N bond); 2) GTA has been almost completely removed from the hydrogels, except for the 1:1 sample, in which the carbonyl peak at 1750 cm^{-1} (belonging to free GTA) is non negligible. The latter result was, however, expected, since in the 1:1 sample the excess of GTA was increased over a reasonably necessary amount to set an upper limit to the use of GTA.

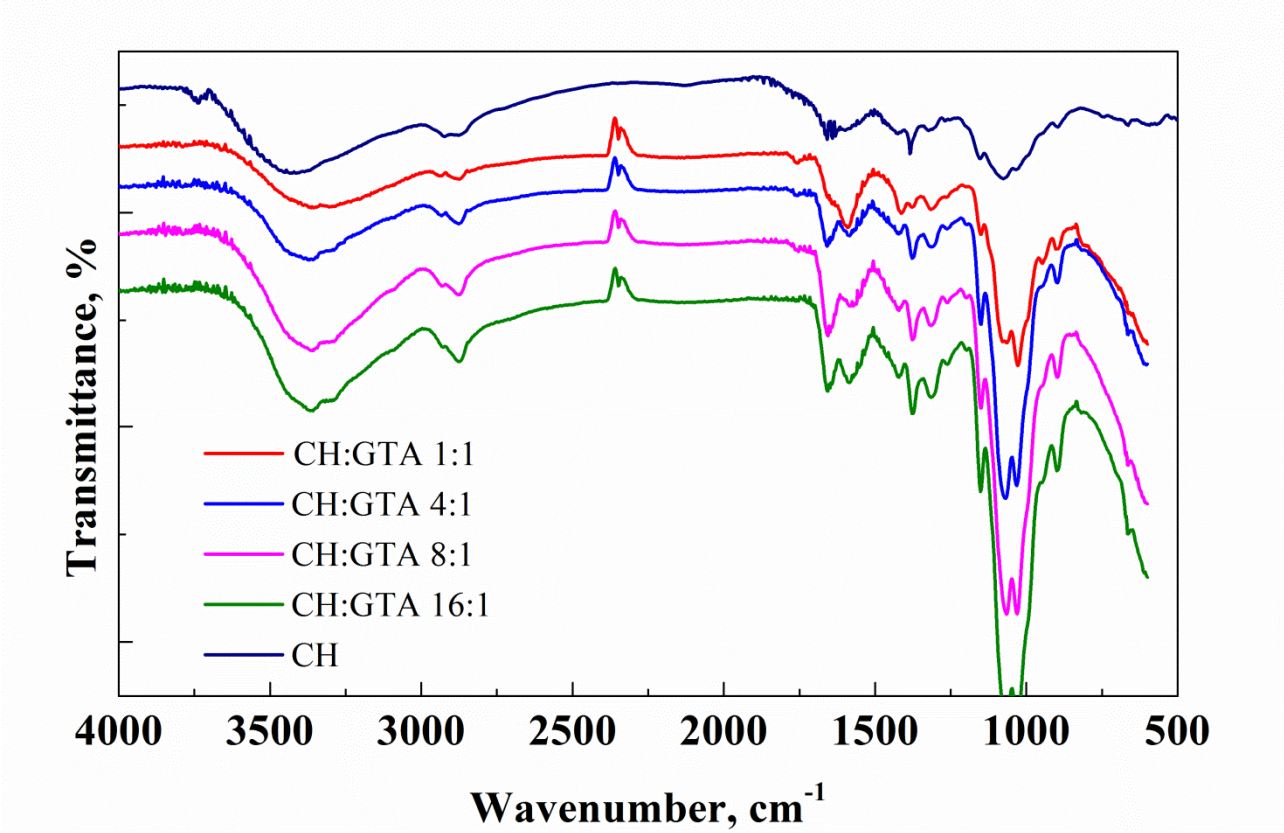

Figure 4. FTIR spectra of pure CH and CH aerogels, 2 % w/w crosslinked with GTA.

3.3 TGA analysis

In Figure 5 and in Table 3, thermogravimetric curves and decomposition temperatures (T_d) of CH aerogels are reported. A comparison between the decomposition temperatures (T_d) shows that CH aerogel has a T_d equal to 218 °C, whereas the crosslinked CH samples show higher T_ds, which increase with the amount of crosslinking agent (from 247 °C for CH:GTA 16:1 to 251 °C for CH:GTA 1:1). These data are consistent with the fact that the crosslinking reaction leads to a more

thermally stable structure, and to a consequent increased degradation temperature of the CH aerogel samples.

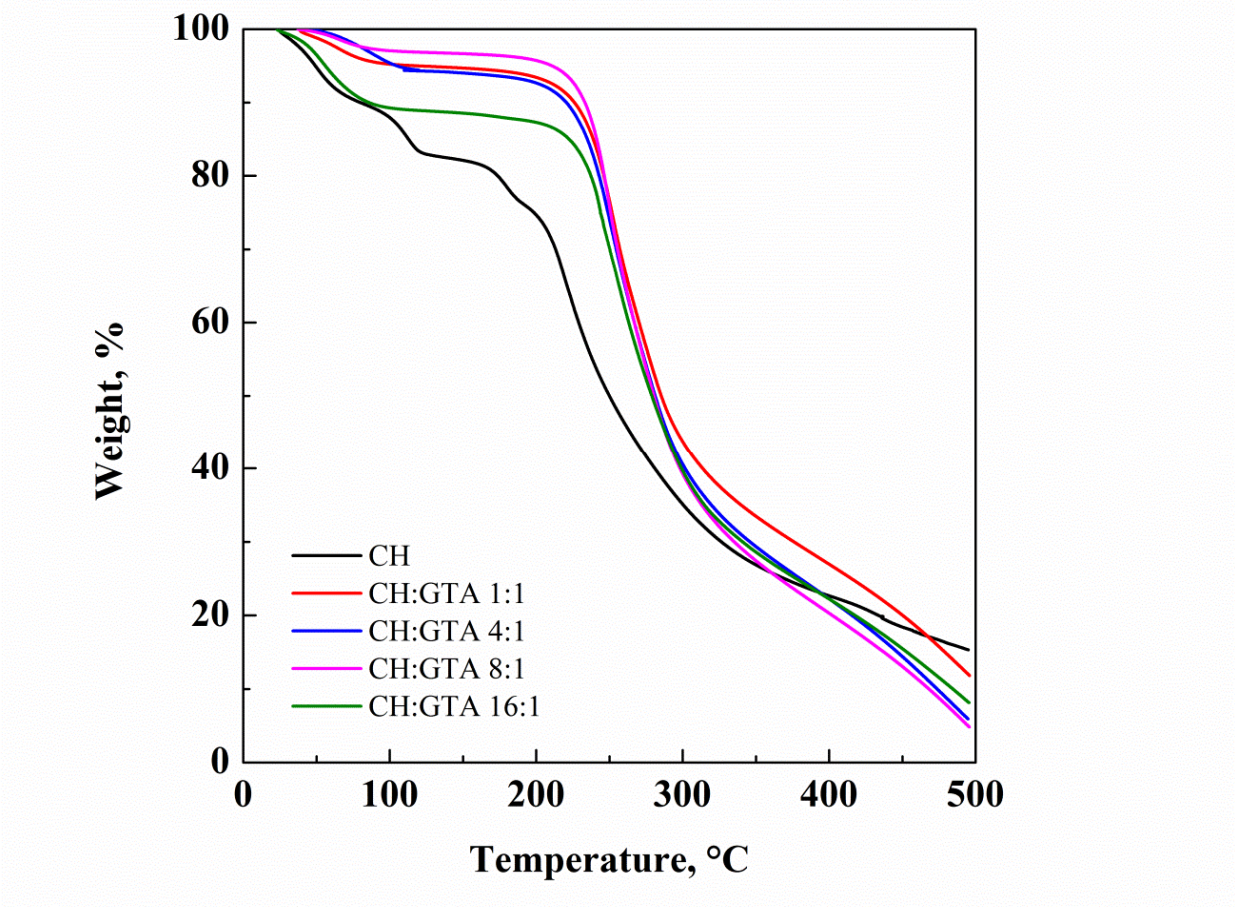

Figure 5. Thermogravimetric analysis of CH aerogel samples.

Aerogel	T_d^*, °C
CH	218
CH:GTA 16:1	247
CH:GTA 8:1	248
CH:GTA 4:1	249
CH:GTA 1:1	251

*T_d = Degradation temperature, corresponding to the maximum weight loss of the sample.

Table 3. Degradation temperature of CH aerogels crosslinked with GTA.

4 Conclusions

SC-CO_2 drying was successfully applied for the attainment of CH aerogels. GTA residues measurements confirm that CH hydrogels crosslinked with GTA were successfully dried, preserving their nanostructure: indeed, no collapse of the nanostructure was observed in the aerogels surface and section. The hypothesis that SC-(CO_2 + ethanol) mixture is able to efficiently extract GTA residues entrapped in the gel matrix was verified and negligible residues of GTA were measured during release experiments.

Therefore, the produced CH aerogels not only maintained their delicate nanostructure necessary for the cells cultivation, but can also be safely used for TE applications.

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