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Supercritical CO_2 generating chitosan devices with controlled morphology. Potential application for drug delivery and mesenchymal stem cell culture

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ABSTRACT

In this work, a novel approach involving supercritical carbon dioxide ($scCO_2$) induced phase inversion technique was developed to produce chitosan devices using moderate temperatures and three very environmentally acceptable solvents (water, ethanol and CO_2). The morphology and three-dimensional (3D) structure were controlled by altering the co-solvent (ethanol) composition in the carbon dioxide non-solvent stream during the demixing induced process. Microarchitectural analysis by scanning electron microscopy identified the production of particulate agglomerates when 10% of ethanol in the $scCO_2$ stream was used and the ability to make porous membranes with different morphologies and mechanical properties depending on the programmed gradient mode and the entrainer percentage (2.5–5%) added to the $scCO_2$ stream. These structures were characterized in terms of pure water flux, porosity, mechanical properties and biodegradability. These chitosan matrices exhibited low solubility at neutral pH conditions, with no further modifications. We also demonstrated that the current method allows for a single-step preparation of an implantable antibiotic release system by co-dissolving gentamicin with chitosan and the solvent. Finally, the cytotoxicity, as well as the ability of these structures to support the adhesion and proliferation of human mesenchymal stem cells (MSC) *in vitro* were also addressed. The studies described may provide a starting point for the "green" design and production of chitosan-based materials with potential applications in tissue engineering and regenerative medicine, as well as drug delivery.

1. Introduction

Biocompatible and biodegradable polymers have various important applications in the biomedical field particularly in the development of supports for tissue repair and regeneration [1]. These structures have a high level of porosity, with a good interconnectivity among the pore network system together with significant mechanical strength and flexibility. Membranes are the most widely studied scaffolds for guided bone regeneration. They are useful for repair in sites where limited mechanical loading exists, for example, in some cranial or maxillofacial areas, in dental applications [2] and as 3D matrices in perfusion bioreactors [3]. Another important area of application of such materials is in drug delivery. In biomedical applications, residual solvents are undesirable since they may contaminate the device and cause toxicity problems. In order to avoid those negative effects, alternative approaches are being developed, namely supercritical fluid processing, of which the most commonly used is carbon dioxide (CO_2). Besides the environmental advantages and the additional parameters that can be used to control the morphology (pressure, temperature, and depressurization rate), $scCO_2$ presents liquid-like densities and gas-like viscosities and diffusivities, ideal for penetrating into porous structures. In addition, being a gas at normal conditions, CO_2 can easily be removed from the pores without leaving any solvent residues. Importantly, $scCO_2$ technology potentially allows the production of sterile, ready-to-use devices, due to the high pressure features of this technique. In fact, reports in the literature suggest that $scCO_2$ can be potentially used for sterilization of biomedical devices, being effective against bacteria [4], viruses [5] and spores [6], although additives are required in order to achieve terminal sterilization, namely hydrogen peroxide [6] or peracetic acid [7]. A complete review concerning the potentialities of supercritical fluids in the processing of polymer systems for drug delivery, as well as tissue engineering/regenerative medicine can be found in literature [8]. During the last decade several techniques were developed for

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the preparation of porous structures with supercritical fluids: gas foaming [9,10], supercritical fluid emulsion templating [11], lithography and CO_2-assisted phase inversion [12–14]. A special focus was given to the application of this technique in the processing of biocompatible polymers [15–18].

In this work, chitosan, a well-known biodegradable, biocompatible and non-toxic natural polymer with possible applications in the biomedical field [19] (*e.g.* dialysis membranes, contact lenses, antitumor uses, drug delivery controlled-release systems and tissue engineering) was processed using a CO_2-assisted phase inversion method. In particular, chitosan-based membranes have been used for medical applications, such as suture thread and artificial skin [20]. Recently, chitosan was used as starting material elected to fabricate biodegradable stents that can deliver drugs locally [21]. The administration of drugs by a device delivery system has advantages over conventional drug therapies. Depending on the appropriate clinical application, a rational design of the system namely concerning the selection of the support material, its morphology and the technique to incorporate the therapeutic molecules is decisive. In this work, we attempted to produce a low cost chitosan porous implantable material that would allow the release of an antibiotic in an affected region. Gentamicin is a widely used antibiotic in the treatment of gram negative infections and its incorporation in a chitosan controlled release device could be useful in the prevention or treatment of wound infections there by facilitating wound healing [22]. In the present study, gentamicin was used as a model molecule which was incorporated into chitosan membranes by mixing it with the polymeric casting solution.

Chitosan has also been proposed as a scaffold to sustain cell adhesion and proliferation for application in tissue engineering [23]. In this context, human mesenchymal stem cells (MSC) from the bone marrow (BM) are a potential source of osteoblasts and chondrocytes for bone and cartilage repair, respectively. Many efforts have been performed to design suitable biomaterials to support MSC adhesion/proliferation, by attempting to mimic the BM *in vivo* microenvironment and maintaining cell multipotency. However, most of the ongoing research is more focused on the controlled differentiation of MSC [24–26], rather than the *ex vivo* expansion/maintenance of these multipotent stem cells. Due to the very low number of MSC present in the BM, a rapid *ex vivo* expansion method is needed to meet the highly demanding dose of MSC for clinical application in terms of cellular therapy (*e.g.* 1–2 million MSC/kg in the settings of hematopoietic stem cell transplantation, enhancing the engraftment and/or preventing/treating graft-*versus*-host disease) [27–29]. Therefore, the successful expansion of MSC under strictly controlled conditions would clearly facilitate their potential clinical uses. Here, chitosan membranes produced by $scCO_2$ technology were evaluated in terms of cytotoxicity following International Standard guidelines, as well as their ability to support MSC adhesion/proliferation *in vitro* under static conditions, envisaging the future use of these devices as scaffolds to sustain stem cell function and proliferation *in vitro* in a highly controlled culture system for application in clinical settings [30,31].

2. Experimental

2.1. Materials

Chitosan (75–85% deacetylated, $M_w = (190–310)\,kg\,mol^{-1}$), absolute ethanol, glacial acetic acid (purity ≥99%), gentamicin sulphate (UPS testing specifications), o-phthalaldehyde (purity ≥97%), sodium 1-heptanesulfonate, thioglycolic acid (purity ≥98%), phenol (purity ≥95%), crystal violet, phosphate buffered saline (PBS), accutase™, methanol and acetonitriline (HPLC grade) were purchased from Sigma–Aldrich. Isopropanol alcohol (purity ≥99%) was purchased from Pronalab and sodium hydroxide (purity ≥99%) from Riedel-De-Haën. RPMI-1640 (a Roswell Park Memorial Institute medium), Dulbecco's modified essential medium low glucose (DMEM-LG), anti-human CD105, trypan blue and fetal bovine serum (FBS) used in cell culture were purchased from Invitrogen. L929 cells were obtained from DSMZ, Germany and WST-1 Proliferation Kit from Roche. Carbon dioxide was obtained from Air Liquide with 99.998% purity. All materials and solvents were used as received without any further purification.

2.2. Membrane preparation

A detailed description of the apparatus and the experimental procedure can be found in previous publications [15,16]. In a typical procedure, the casting solution, normally 4% (w/w) of polymer in acidified water (1% acetic acid) is loaded into a stainless steel cap (with a diameter of 68 mm and 1.5 mm height) and placed inside the high pressure vessel. The cell is then immersed in a visual thermostated water bath, heated by means of a controller (Hart Scientific, Model 2200) that maintains the temperature within ±0.01 °C and a non-solvent flow is added until the desired pressure is reached, with an exact flow, using two Gilson piston pumps model 305 and 306. The non-solvent is a binary mixture of ethanol and CO_2 with different compositions from 2.5 to 10% of ethanol. The ethanol addition mode to the non-solvent stream is a key parameter to control membrane morphology. Fig. 1 presents two variants that were tested in this work: introduction of a co-solvent (ethanol) in the non-solvent with variable composition (gradient mode) and with a constant composition (isocratic mode).

After reaching the normal operational pressure (20 MPa), the supercritical solution passes through a back pressure regulator (Jasco 880-81) which separates the CO_2 from the acidified water used in the casting solution. The pressure inside the system is monitored with a pressure transducer (Setra Systems Inc., Model 204) with a precision of ±100 Pa.

All the experiments were performed at 20 MPa and 60 °C with a non-solvent (CO_2 + ethanol) flow of 4.9 g/min during 6 h. After this time period, a pure CO_2 flow is passed through the high pressure cell to remove the ethanol. At the end, the system is slowly depressurized during 10 min and a thin homogeneous membrane is obtained.

2.3. Membrane characterization

Membranes were characterized using scanning electron microscopy (SEM) in a Hitachi S-2400, with an accelerating voltage set to 15 kV. Then membrane samples were frozen and fractured in liquid nitrogen for cross-section analysis. All samples were coated with gold before analysis. The tensile properties of the membranes were tested with a tensile testing machine (MINIMAT firm-ware v.3.1) at room temperature. The samples were cut into strips with 2 mm × 15 mm. The length between the clamps was set at 5 mm and the speed of testing set to 0.1 mm/min. A full scale load of 20 N and maximum extension of 90 mm were used. Measurements were performed with dried membranes, as well as membranes soaked in PBS solution for 1 h before testing. Load extension graphs were obtained during testing and converted to stress strain curves applying the following equations:

$$\text{Stress} = \sigma = \frac{F}{A} \quad (1)$$

$$\text{Strain} = \varepsilon = \frac{\Delta l}{L} \quad (2)$$

where F is the applied force; A the cross-sectional area; Δl the change in length; and L is the length between clamps.

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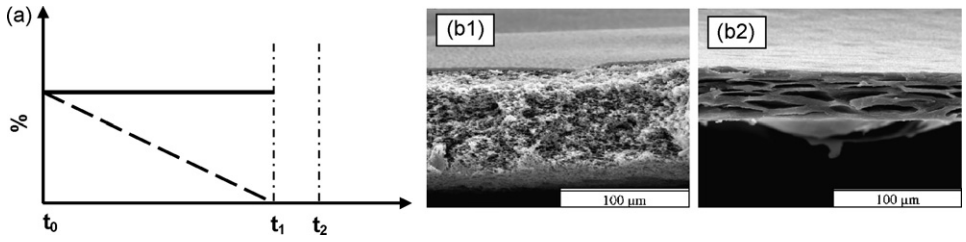

Fig. 1. (a) Different operational modes that can be used to introduce the co-solvent (ethanol) in the non-solvent (CO_2) stream. Hatched line-gradient mode; SEM pictures of chitosan membranes prepared (b1) isocratic mode and (b2) gradient mode.

The permeability to pure water was determined by measuring the water flux through the membranes using a 10 mL filtration unit (Amicon Corp., model 8010) with an effective area of 4.1 cm^2. All the experiments were carried out varying the applied hydrostatic pressure from 0 to 5 bar. At least three clean water flux measurements were performed for each membrane.

The porosity of the membrane was determined by mercury porosimetry (micromeritics, autopore IV).

2.4. Membrane degradation studies

The biodegradability of chitosan films *in vitro* was studied by degrading chitosan structures in lysozyme solution (Lys) [32] and PBS (pH 7.4). After specific time intervals, the films were taken out from the solutions, washed with distilled water and freeze-dried. The weight was measured before and after lyophilization.

2.5. Drug loading

Gentamicin was loaded into the chitosan membranes by co-dissolving it with the polymer in acidified water. Different amounts of drug, 40, 80 and 160 $mg_{gentamicin}/g_{chitosan}$ were used. The membranes were prepared using 2.5% (w/w) ethanol isocratic mode in the non-solvent.

For the controlled release studies, a membrane portion, with approximately 3 cm^2, was immersed in 25 mL of PBS solution (pH 7.4), at 37 °C and 1 mL aliquots were withdrawn at predetermined time intervals. The samples were analysed by HPLC (Knauer Smartline Pump 1000 combined with a Low Pressure Gradient, autosampler and UV/Vis SmartLine 2500). To determine the amount of gentamicin, a derivatization procedure was applied according to the USP reference standards. Briefly, the o-phthalaldehyde (OPA) reagent was prepared by adding 1 g of OPA to 5 mL of methanol, and 95 mL of boric acid solution 0.4 mol/L, previously adjusted with 8N sodium hydroxide to a pH of 10.4, and 2 mL of thioglycolic acid. The resulting solution was adjusted with 8N sodium hydroxide to a pH of 10.4. Samples were prepared by adding 1 mL of gentamicin solution, 0.5 mL of isopropyl alcohol and 0.4 mL of OPA reagent, mixing and adding isopropyl alcohol to obtain 2.5 mL of solution. The mixture was heated at 60 °C for 15 min. The mobile phase was prepared adding 700 mL of methanol, 250 mL of water, 50 mL of glacial acetic acid, 5 g of sodium 1-heptanesulfonate. A C18 column (Knauer) was used in isocratic conditions with a flow rate of 1.4 mL/min and a volume of injection 20 μL. Quantification was performed at 330 nm. The total mass of released drug was calculated taking into account the aliquots taken.

2.6. Cytotoxicity assay

Chitosan membranes were tested for cytotoxicity following the ISO 10993-5 guidelines. Briefly, triplicates of 1 cm^2 membranes were placed in polystyrene tubes containing 3 mL of RPMI—1640 with 10% (v/v) of FBS and kept in an incubator (37 °C, 5% CO_2, fully humidified) for 3 days. The liquid extracts were used to culture L929 mouse fibroblasts (initial density 80×10^3 cells/cm^2) in 24-well plates for 2 days. The cell metabolic activity was determined by analysing the conversion of WST-1 (light red) to its formazan derivative (dark red—absorbance at 450 nm after a 2.5 h incubation at 37 °C) using a WST-1 Cell proliferation kit. The results were normalized to the negative control for cytotoxicity (fresh RPMI medium) and compared to the positive control (0.01 M phenol).

In order to evaluate the effect of the direct interaction between chitosan membranes and L929 cells, membranes were conditioned with RPMI medium (2 cm^2/mL) overnight before cell seeding in 24-well plates (6000 cells per well). At day 3 of culture, cells cultured on the membranes were stained with crystal violet and then observed under an inverted light-phase microscope (Olympus) in order to qualitatively evaluate the morphology, cell-to-cell contact and attachment. Briefly, cells were washed with PBS solution and then stained with crystal violet (0.5%, w/v in methanol) for 30 min, washed three times with PBS solution and then observed.

2.7. Mesenchymal stem cell cultures

Heparinized human bone marrow (BM) was obtained from healthy donors after informed consent. BM mesenchymal stem cells (MSC) were isolated as previously described [33] and kept cryopreserved in liquid nitrogen until further use. Passage 3 MSC (negative for the hematopoietic markers CD14, CD34, CD45 and over 90% positive for CD73, CD90 and CD105, assessed by flow cytometry) were seeded on top of chitosan membranes (2.5% ethanol, isocratic mode—Fig. 2a), previously conditioned overnight at 37 °C with DMEM-LG supplemented with 10% FBS (MSC qualified), at a cell density of 3000 cells/cm^2 using 24-well plates (in triplicate). Medium was replaced twice a week within the 2-week culture period. At day 7 and 15, cells were harvested with 0.5 mL accutase™, counted and characterized in terms of cell viability using the trypan blue exclusion dye test under the inverted light-phase microscope. A control condition without membranes was also performed by culturing cells on traditionally used tissue culture wells made of polystyrene.

In order to evaluate the phenotype of the cells cultured on the chitosan membranes, an anti-human CD105 monoclonal antibody was used, with an appropriate anti-IgG isotype control. At each time point, cells were stained with anti-CD105 and analysed in a FACScalibur equipment (Becton Dickinson). In addition, cells were characterized in terms of clonogenic ability by performing colony forming-unit fibroblast (CFU-F) assays. At day 0 and 7, cells were plated at a density of 10 cells/cm^2 in T-25 flasks (250 cells per flask) and cultured for 2 weeks, without medium replenishment. They were then stained with crystal violet (0.5%, w/v in methanol) for 30 min, washed three times with PBS solution and observed under the microscope. Only colonies with more than 50 cells were considered.

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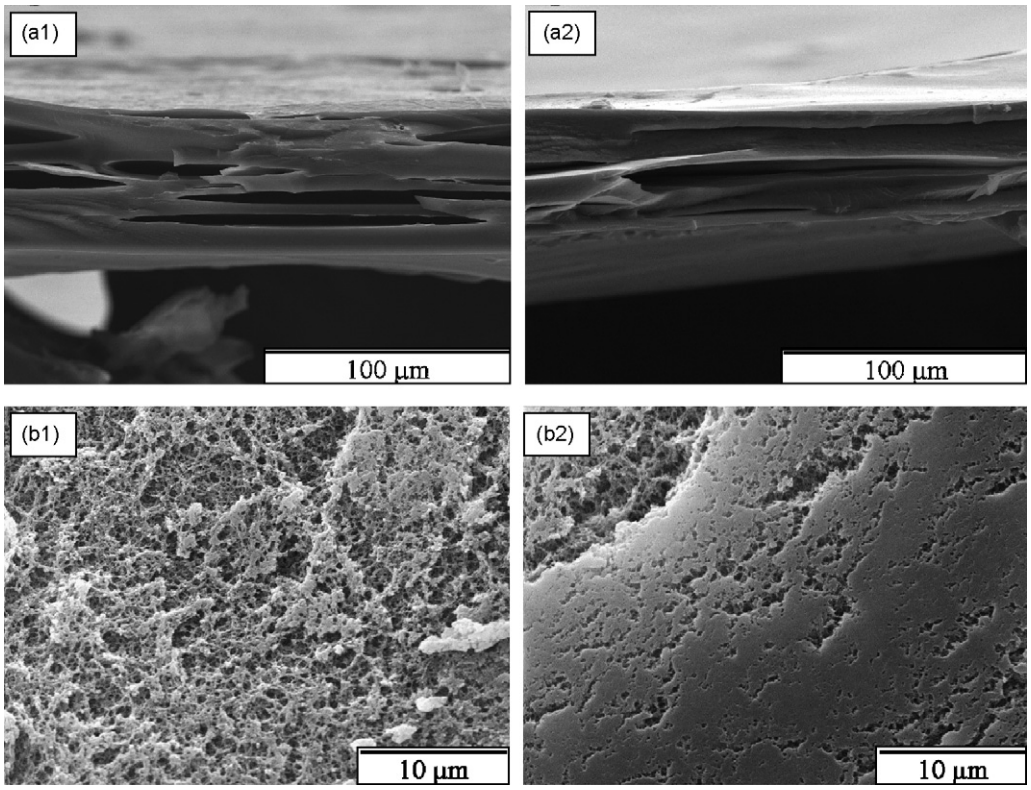

Fig. 2. SEM pictures of the membranes cross-section prepared with different co-solvent compositions: (a1) 2.5% gradient; (a2) 2.5% gradient; (b1) 10% isocratic; (b2) 10% gradient.

3. Results and discussion

3.1. Beads/membranes production

In this study a new strategy was proposed to produce porous chitosan structures (membranes and particles) using supercritical carbon dioxide ($scCO_2$) processing. The production of porous structures using a CO_2-assisted phase inversion method has some requirements. The first one is the solubilization of the polymer in a solvent and secondly the solubility of this solvent in $scCO_2$ that will act has non-solvent and will induce the precipitation of the polymer. The widespread use of $scCO_2$ to process non-modified chitosan is limited due to the fact that this polymer is only soluble in acidified water which is a solvent with very little affinity/solubility in $scCO_2$. To overcome this limitation it was necessary to use an entrainer (ethanol) in the supercritical fluid to increase the mutual affinity between solvent and non-solvent. The group of solvents used in this work (carbon dioxide, water and ethanol) are three of a short (and ever-diminishing) group of solvents that may be used without restrictions and for this reason ideal for the generation of porous structures. Reverchon et al. have used a similar approach, ethanol expanded by $scCO_2$, to prepare poly (vinyl alcohol) membranes [34]. In this work the amount of ethanol was minimized in order to maximize the advantages of using supercritical fluid technology.

In a previous publication focusing membrane production using $scCO_2$ processing [15], Temtem et al. reported that the affinity between solvent and non-solvent was a key parameter to modulate the morphology of the membrane in terms of pore size distribution and also interconnectivity. As the mutual affinity between solvent and $scCO_2$ decreases, the membrane porosity and the average pore size increases. The 'tunable' solvent power of the supercritical fluid, owing to its variable solvent density (dependent on pressure and temperature) and viscosity, is an important advantage when compared to traditional solvents. In this particular case, the mutual affinity between the solvent and non-solvent is very low since water and CO_2 are from two completely different natures. To overcome this drawback, a co-solvent or entrainer was added to the non-solvent following an isocratic or a gradient mode (Fig. 1a). The introduction of the co-solvent is crucial to control the morphology, as it is shown in Fig. 1(b1 and b2), where the comparison of the two SEM images clearly shows the differences observed when changing from gradient to isocratic mode. When the gradient mode was used (Fig. 1b2) starting from 5% ethanol at $t = 0$ min and ending with 0% ethanol at $t = 300$ min, membranes with large pores were obtained; however, when the co-solvent composition remained constant the pore size diameter decreased. Similar results were observed by changing the ethanol composition from 2.5 to 10% (Fig. 2). When the ethanol ratio reaches a certain level, particle agglomerates were obtained (Fig. 2b), highlighting the broad potential of this technology. Overall, the CO_2 induced phase inversion technique can create porous structures in a membrane shape, but also particle agglomerates or beads with interesting potential applications. Membranes produced by isocratic and gradient mode exhibited structures with low porosity, 40–30%, respectively, while particle agglomerates present higher levels of porosity around 65–50%. The decrease in porosity from isocratic *versus* gradient mode can be attributed to the different morphologies that were obtained. The ethanol composition in the non-solvent stream and operational mode used, isocratic or gradient, and the resulting morphology and water permeability of different chitosan structures are summarized in Table 1. Clearly, permeability through a porous material is an important parameter for its function, particularly considering the operation of a polymeric membrane under dynamic conditions. Fluid flow through the membranes decreased when the addition mode of ethanol changed from gradient to isocratic, in agreement

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Table 1
Influence of non-solvent composition in devices morphology and water permeability.

% ($w_{ethanol}/w_{total}$)	Mode	Morphology	Water flux (L/(m^2 h bar))
2.5	Isocratic	Porous membrane	1.02 ± 0.19
2.5	Linear gradient	Porous membrane	0.18 ± 0.01
5	Isocratic	Porous membrane	0.42 ± 0.03
5	Linear gradient	Porous membrane	0.39 ± 0.05
10	Isocratic	Particle agglomerates	N/A
10	Linear gradient	Particle agglomerates	N/A

with the relative membrane porosities. These structural differences are related with the thermodynamics and kinetics of the phase inversion process. The richness of the solvent–non-solvent interactions and operational conditions that can be selected to control for the solubilization or precipitation of polymer structures is represented in a simplified hypothetical quasi-ternary phase diagram for the system polymer–solvent–non-solvent (see Fig. 3 and its caption). All possible composition combinations of the components can be plotted in a triangle. The corners represent each intervenient: (i) the polymer–chitosan; (ii) the solvent–acidified water and (iii) the non-solvent–($scCO_2$ + ethanol); the axes represent the three pseudo-binary systems and a point in the triangle is the overall mixture. In the liquid + fluid region a primary envelope (binodal curve) and a secondary envelope (spinodal curve) enclose demixing boundary. Both curves coincide at the critical point and the region between them corresponds to a metastable state. The phase diagram is pressure and temperature dependent.

As far as liquid–liquid demixing of polymer solutions is concerned, two different mechanisms have to be considered: nucleation/growth and spinodal decomposition [35]. The type of structures obtained and pore dimension depend on the path followed through the ternary diagram [18,36]. Nucleation and growth (usually a slow process) is the expected mechanism when a system leaves the thermodynamically stable condition and slowly enters the metastable region (region between the spinodal and the binodal) and can be represented in the ternary diagrams by the two distinguished paths (please refer to Fig. 3): path (4) – when demixing is started somewhere below the gas–liquid critical point – nucleation and growth of concentrated phase (polymer-rich phase) occurs. Polymer-rich phase is nucleating in polymer-poor phase. Therefore, low-integrity powdery agglomerates would be produced as it is exemplified in Fig. 2b where chitosan powder agglomerates can be observed.

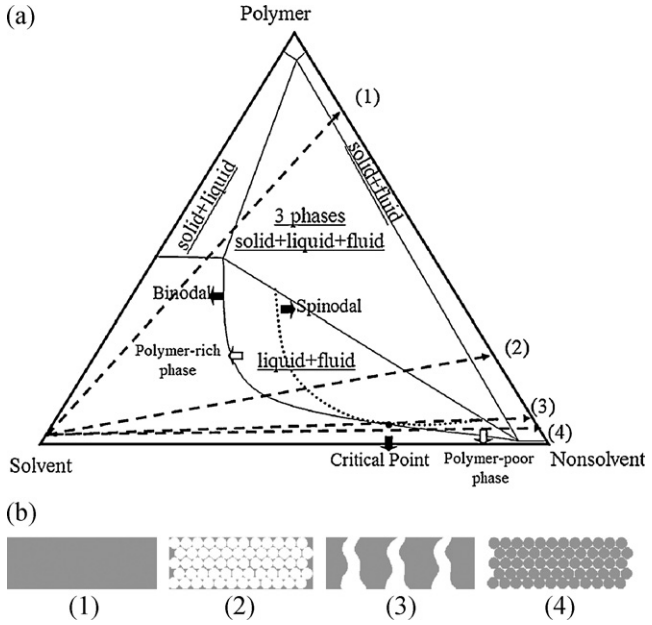

Fig. 3. Hypothetical ternary phase diagram for the system polymer–solvent–non-solvent. (a) Composition paths that can occur during membrane formation; (b) possible structure formation in the different paths of the gelation/vitrification region and liquid–liquid demixing gap: dense structure formation (1), cellular morphology due to nucleation and growth of the polymer-rich phase (2), bicontinuous morphology due to spinodal decomposition (3) microparticles due to nucleation and growth of a polymer-poor phase (4).

Considering path (2) – when demixing starts somewhere above the critical point – nucleation and growth of diluted phase (polymer-poor phase) occurs. Polymer-poor phase is nucleating and growing in polymer-rich phase. The former pathway originates porous structures while the latter one results in a porous solid matrix as it is exemplified in Fig. 2a.

The second mechanism, much less considered in literature, is spinodal decomposition that takes place in a fast quench into the two phase region limited by the spinodal curve or even in a slower transition crossing the metastable region near the critical point. In this case, phase separation initiates with concentration fluctuations of increasing amplitude, giving rise to two continuous phases (path 3). An example of this type of membranes was presented by Reverchon and Cardea [14] who prepared cellulose acetate membranes using a supercritical fluid assisted process. Finally, it is also possible to obtain a membrane when polymer molecules solidify by gelation or crystallization giving rise to the formation of dense structures (path 1).

Chitosan devices were characterized in terms of mechanical properties and biodegradability. Tensile tests provide an indication of the strength and elasticity of the membranes which are important considering their potential applications; for instance, it is suggested that films suitable for wound dressing should be preferably strong, though flexible [37]. Fig. 4 presents the stress–strain curves under dry and wet conditions. Under dry conditions (Fig. 4a) films revealed an elastic behavior for 8% strain and after this value the stress exceeds a critical value, undergoing plastic, or irreversible, deformation. Membranes prepared with gradient mode exhibited a very high stress break (50–70 MPa, for 2.5 and 5% ethanol, respectively) in accordance with literature values [38,39] while the ones that were produced with isocratic mode 2.5 and 5% revealed the ability to undergo a longer elongation (22–24%) at the break, probably due to their higher porosity. Under wet conditions (Fig. 4b) membranes revealed an exclusive elastic behavior with appreciably different stress–strain behavior relative to the dry samples with higher elongation values (50–75%) and a lower break stresses (8–16 MPa) also in agreement with the data from literatures [38,39]. The different mechanical properties allow us to conclude that CO_2-assisted phase inversion is able to prepare membranes with a wide range of tensile properties.

In the human body, chitosan is mainly degraded by lysozyme and its *in vitro* biodegradability is generally investigated by using a lysozyme containing PBS solution (LysS) [32]. Our results in Fig. 5 show that the supercritical processed devices have different degradation profiles when immersed in LysS or in PBS solution. Analysing Fig. 5a we can observe that after 10 days membranes kept 95% of

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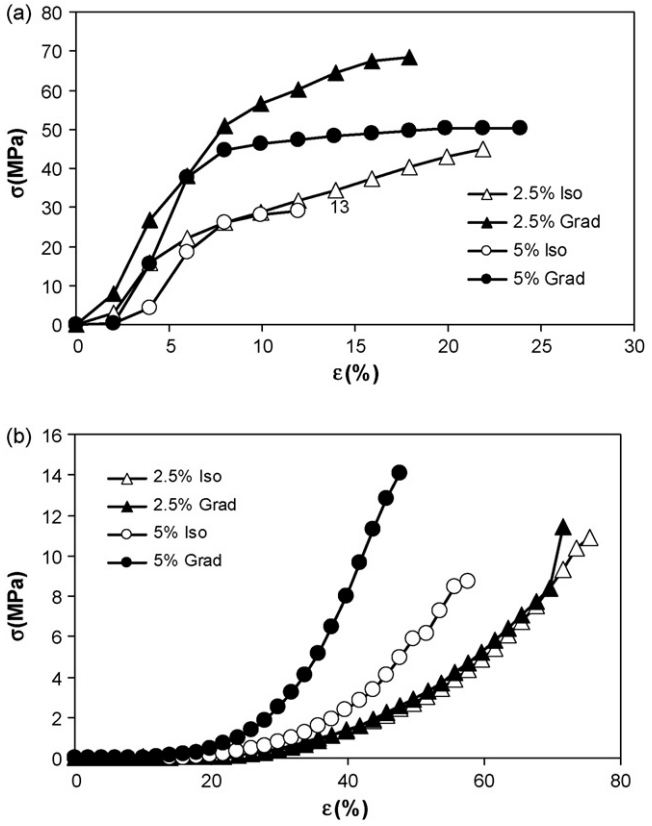

Fig. 4. Stress–strain curves for chitosan membranes under (a) dry conditions and (b) wet conditions (soaked in PBS for 1 h).

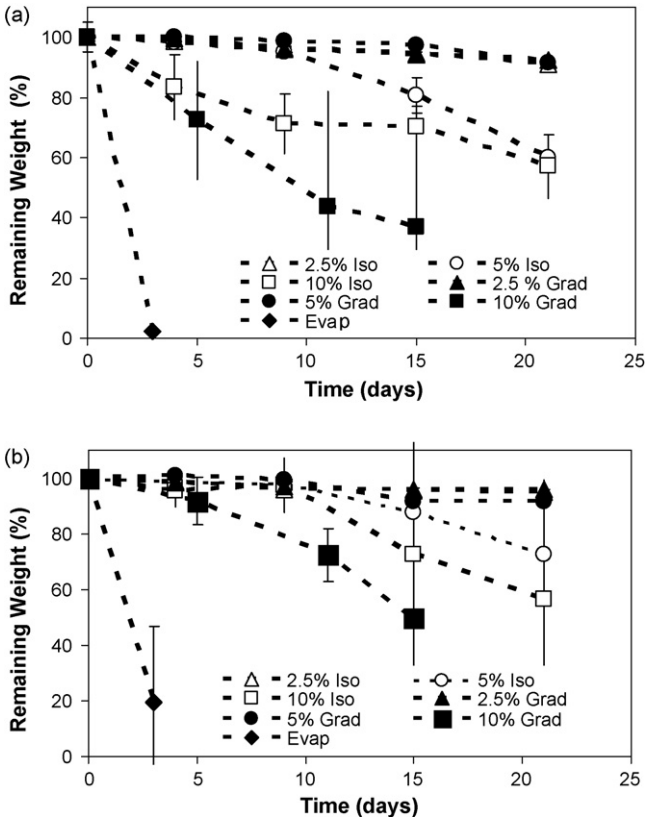

Fig. 5. Degradation of the different chitosan porous structures. (a) Lysozyme solution and (b) phosphate buffer solution.

its initial weight, while for the particle agglomerates there was a loss of 30%. The higher porosity of the latter structures facilitates enzyme access to chitosan. Longer immersion periods lead to faster degradation rate of membranes prepared with 5% ethanol (isocratic mode) and 10% ethanol (either isocratic or gradient mode), and after 20 days of immersion, all these devices had lost approximately 50% of its initial weight. However, the ones produced with 2.5% ethanol kept more than 90% of their initial weight. In terms of immersion in PBS (Fig. 5b), after a 10 days period, it is possible to observe a negligible loss of weight for the structures that were prepared by the CO_2-assisted phase inversion method contrary to the membrane that was prepared by evaporation, that shows a completely loss of weight after a 3 days immersion. Chitosan membranes prepared with 2.5% ethanol, either following isocratic or gradient mode kept more than 95% of their weight even after a 20 days immersion period but other chitosan structures degraded faster, loosing 30–50% of its weight after the same time period. Usually chitosan dissolution of scaffolds or membranes is prevented by rehydrating samples in either diluted NaOH, or in an ethanol series. In our methodology, $scCO_2$ is able to remove the acetic acid vestiges and prepare structures without additional post-treatment processes avoiding some of the disadvantages of the post-treatments: for instance, scaffolds hydrated in NaOH exhibited some shrinkage and distortion, probably caused by base-induced changes in crystallinity and associated structural stresses [40]. Another possibility is the use of reticulation procedures, but since crosslinking agents are usually toxic compounds that might detrimentally affect bioactive substances or cells attached to the surface of the material [41], therefore these were avoided in this work.

3.2. Drug release devices

Apart from their biological activity, one of the more important properties of polysaccharides, in general, is their ability to form hydrogels and their application as drug release devices. $ScCO_2$ has the potential to be an excellent environment to form controlled release systems [42]. In this work, gentamicin was used as a model drug, being incorporated into the membrane by bulk loading in the casting solution. The membrane prepared with 2.5% ethanol isocratic mode was selected for these particular studies due to the relative high porosity and water permeability. The mechanical properties, specially the high elasticity of this particular membrane type was also an important feature for this particular choice.

Finally, Fig. 6 shows the release profiles exhibited by membranes loaded with different amounts of gentamicin. Three different loadings were introduced in the casting solution. After membrane preparation the release profiles were determined using mem-

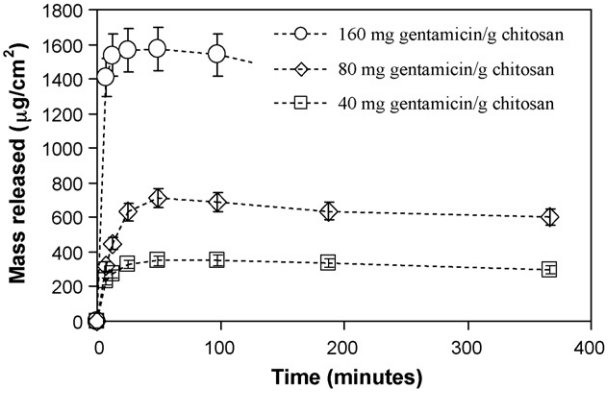

Fig. 6. *In vitro* drug release profiles for chitosan membranes loaded with 40, 80 and 160 $mg_{gentamicin}/g_{chitosan}$.

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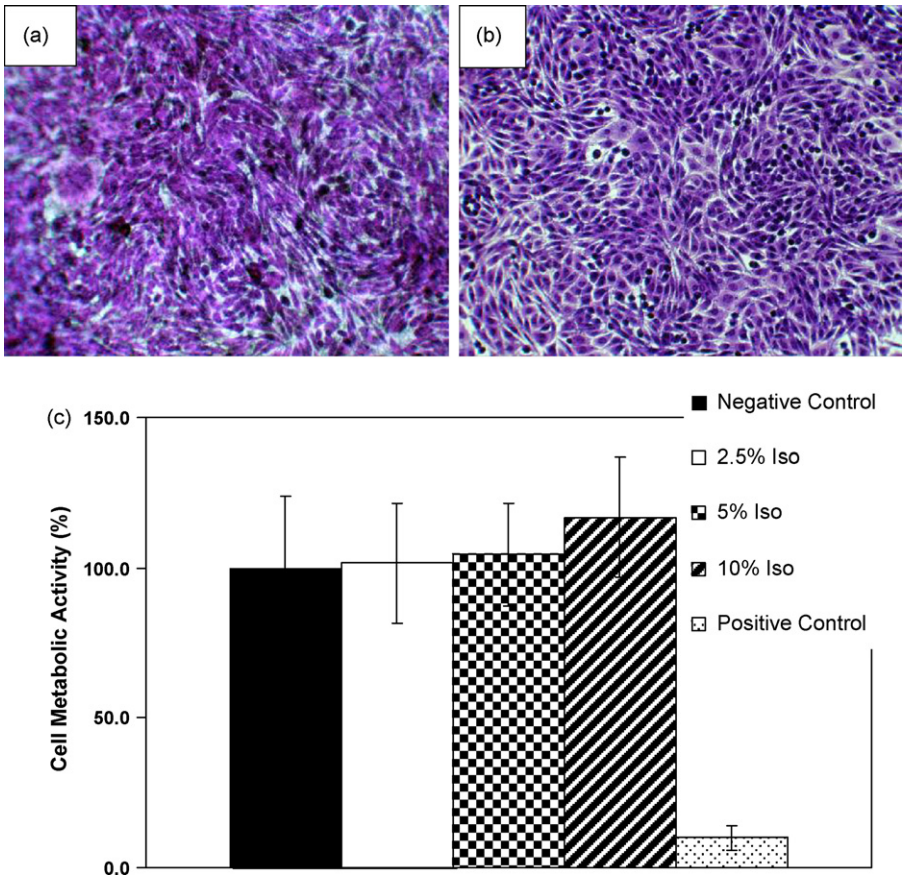

Fig. 7. Representative images of L929 fibroblast cells cultured on chitosan membranes (2.5% isocratic) (a) and polystyrene control (b) after 3 days in culture, presenting their characteristic morphology. (c) Cytotoxicity tests of the chitosan membranes following the ISO standards for biomaterials. Negative control: tissue culture plate control (polystyrene); Positive control: 0.01 M phenol.

brane areas of approximately 3 cm^2. The total of drug released was proportional to the amount of drug initially dissolved with the polymer into the casting solution which allows us to confirm that the amount of drug released can be easily controlled by the amount of drug that it is loaded. Membranes prepared with 40 and 80 $mg_{gentamicin}/g_{chitosan}$ released 90% of its gentamicin in PBS at 37 °C in 25 min with a total dosage of 329 and 630 $\mu g/cm^2$, respectively, while the membrane loaded with 160 $mg_{gentamicin}/g_{chitosan}$ displayed the 90% release in only 7 min. These profile features are due to the nonexistence of a crosslinking procedure with a reticulation agent (*e.g.* glutaraldehyde). Indeed, when Thacharodi and Panduranga Rao [43] reported the preparation of chitosan membranes and their use has as transdermal delivery system for nifedipine, they observed higher rates in the membranes that were not crosslinked and attributed this to the higher permeability of this type of membranes. These results might be explained by the inexistence of chemical bonds between the drug and the polymer and the prevalence physical interactions as reported by Reverchon et al. [44]. Another possible explanation is the high swelling degree of these membranes, around 260% ((weight after 24 h immersion-dried weight)/dried weight × 100) in aqueous physiological conditions which makes the drug highly accessible and fastenly released. $ScCO_2$ process was able to efficiently prepare an implantable membrane incorporating a small antibiotic molecule, gentamicin, with a broad dosage range (up to 160 $mg_{gentamicin}/g_{chitosan}$) within a 3D stable and controlled morphologic matrix. However, 160 $mg_{gentamicin}/g_{chitosan}$ is by no means an upper limit, as incorporation in the bulk solution is only limited by the chitosan and gentamicin solubilities in the acidified water.

3.3. Cytotoxicity assay

The use of biomaterials in the settings of tissue engineering and regenerative medicine requires a cytotoxicity evaluation to test cell responses when interacting with the material. Chitosan membranes prepared using different ethanol concentrations – isocratic mode (2.5, 5 and 10% – Fig. 1b2, 2a1 and 2b1) were tested.

In Fig. 7a and b it is possible to observe that cells cultured on chitosan membranes presented their characteristic fibroblastic morphology. Fig. 7c presents the metabolic activity of the L929 cells (normalized to control) after 48 h of culture with medium extracts of the different structures. No cytotoxic effect was observed for any of the chitosan membranes prepared, when compared to commonly used tissue culture grade polystyrene (negative control). These results are in agreement with previously reported data attesting the non-cytotoxicity of chitosan-based materials [45]. Therefore, the non-cytotoxicity of chitosan membranes enables their potential use as scaffolds to sustain human stem cell adhesion and proliferation *in vitro*.

3.4. MSC adhesion and proliferation

Although MSC have been studied for more than 20 years, only recently their true potential for clinical use is being explored. The use of MSC in Cellular Therapy, taking advantage of their trophic and immunoregulatory activities is now considered a practical procedure in the clinical field with promising results, whereas tissue engineering approaches using MSC as starting cell source for their controlled differentiation to form bone and cartilage stands at a

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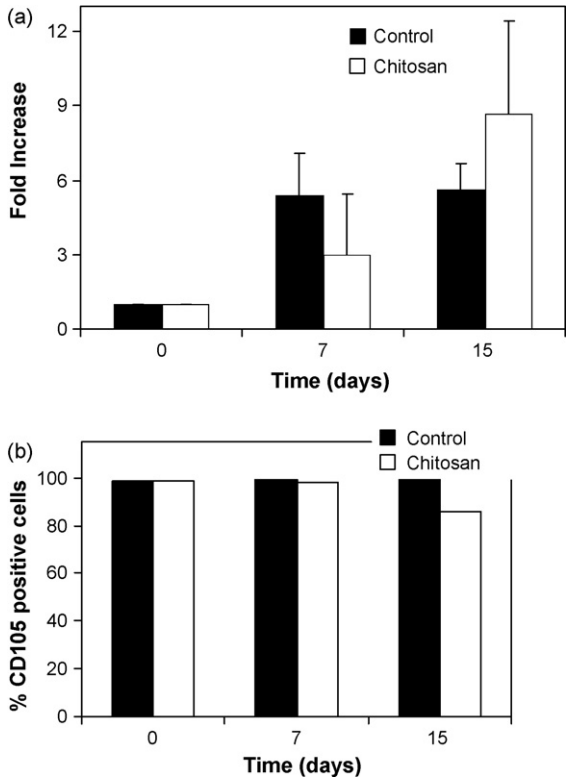

Fig. 8. Output of MSC culture on chitosan membranes (2.5% isocratic) in terms of fold increase in total cell number (a) and CD105 expression (%) (b) in comparison to cells cultured on tissue culture plate control (polystyrene).

research level [29]. Therefore, it is of great interest to have a suitable material that would allow successful MSC expansion *in vitro* to obtain clinically relevant numbers.

Results presented in Fig. 8a indicate that 2.5% ethanol (isocratic mode) chitosan membranes allowed successful MSC adhesion and proliferation, similar to the control condition during the 2-week culture period (9 *versus*. 6-fold increase at day 15). Due to limited cell numbers, immunophenotypic analyses at day 7 and 15 were performed only for CD105 expression. Expanded cells maintained their characteristic immunophenotype, with over 85% positive cells for CD105. CD105 (endoglin) is typically expressed by endothelial and hematopoietic cells [46] but also by MSC, which are consistently negative for endothelial and hematopoietic markers [29]. Endoglin contains the three peptide motif Arg-Gly-Asp (RGD) in its extracellular domain which is associated with the extracellular matrix protein fibronectin. The slight decrease observed in the expression of CD105 by cells cultured on chitosan membranes compared to control condition (86 *versus* 98%) might be due to the need to expose cells attached to the membranes to a longer period with the enzymatic agent (accutase) in order to harvest the cells (2 cycles of 10 min incubation at 37 °C were needed). It was previously reported that a prolonged exposure of cells to proteases is potentially harmful and/or might damage surface cell receptors, namely by decreasing the number of functional integrins available [47]. Ongoing studies testing a range of different scaffolds impregnated/coated with chemomechanical polymers, *e.g.* poly(N-isopropylacrylamide), to acquire direct response to pH levels or temperature changes are being performed. In particular, the incorporation of a thermoresponsive polymer in the scaffold should provide other possibilities such as controlling cell adhesion/detachment, facilitating cell harvesting upon cell expansion *in vitro* [48].

The clonogenic potential of the cultured cells as assessed by the CFU-F assay at day 7 showed 37 and 43 colonies respectively for the chitosan expanded cells and control expanded cells.

Overall, it is envisaged that chitosan membranes might represent suitable structures to support MSC expansion *in vitro*.

4. Conclusions

In summary, we were able to make stable and sterile chitosan porous devices with controlled morphology using the more sustainable supercritical carbon dioxide induced phase inversion process. The results here obtained showed that by changing process conditions we can modulate cell size and membranes morphology from porous membranes to highly porous microparticles and decrease degradation rates of chitosan-based structures *in vitro* physiological conditions by processing them with $scCO_2$. We also used the new process to incorporate therapeutic quantities of gentamicin into the 3D structures. The methodology described in these studies provide a starting point for the design and production of polymeric molded scaffolds, that will be able to incorporate other biologically active molecules such as adhesion molecules and growth factors.

We have demonstrated that the supercritical CO_2-induced phase inversion technique can be harnessed in the field of solid-state porous biomedical materials to make different chitosan devices with a potential to be used as drug delivery systems and scaffolds to sustain *in vitro* cell adhesion and proliferation for possible applications in tissue engineering and regenerative medicine. In particular, we envisage the future use of these stable and sterile, ready-to-use, porous chitosan devices, with defined morphology and pore size, as scaffolds for expansion of human stem cells *in vitro* under highly controlled culture conditions [30,31].

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