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# Supercritical $CO_2$ technology for one-pot foaming and sterilization of polymeric scaffolds for bone regeneration☆

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ABSTRACT

Sterilization is a quite challenging step in the development of novel polymeric scaffolds for regenerative medicine since conventional sterilization techniques may significantly alter their morphological and physicochemical properties. Supercritical (sc) sterilization, *i.e.* the use of $scCO_2$ as a sterilizing agent, emerges as a promising sterilization method due to the mild operational conditions and excellent penetration capability. In this work, a $scCO_2$ protocol was implemented for the one-pot preparation and sterilization of poly(ε-caprolactone) (PCL)/poly(lactic-co-glycolic acid) (PLGA) scaffolds. The sterilization conditions were established after screening against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) vegetative bacteria and spores of *Bacillus stearothermophilus*, *Bacillus pumilus* and *Bacillus atrophaeus*. The transition from the sterilization conditions (140 bar, 39 °C) to the compressed foaming (60 bar, 26 °C) was performed through controlled depressurization (3.2 bar/min) and $CO_2$ liquid flow. Controlled depressurization/pressurization cycles were subsequently applied. Using this $scCO_2$ technology toolbox, sterile scaffolds of well-controlled pore architecture were obtained. This sterilization procedure successfully achieved not only SAL-6 against well-known resistant bacteria endospores but also improved the scaffold morphologies compared to standard gamma radiation sterilization procedures.

## 1. Introduction

Regenerative medicine is a multidisciplinary field that seeks for the development and manufacturing of scaffolds, *i.e.* tridimensional synthetic grafts to guide and restore the tissue growth. Scaffold-associated infections are a common healthcare scenario resulting in severe complications. This situation can be mitigated by strengthening the aseptic practices and improving the methods for scaffolds sterilization (Rochford et al., 2012; Li and Webster, 2017; Arciola et al., 2018).

The sterilization treatment of medical devices must ensure a sterility assurance level (SAL-6) against bacterial endospores prior to their usage, according to the current legal framework (ISO 14937:2009, 2009; Rutala et al., 2008). This value expresses that the probability of viable microorganisms presence in a product after its sterilization must be

*Abbreviations:* ANOVA, analysis of variance; APIs, active pharmaceutical ingredients; ATCC, American Type Culture Collection; CECT, Spanish type culture collection; CFU, colony-forming unit; DMEM, Dulbecco's Modified Eagle Medium; EtO, ethylene oxide; FBS, fetal bovine serum; FDA, Food and Drug Administration; GPC, gel-permeation chromatography; HPLC, high-performance liquid chromatography; ISO, International Organization for Standardization; MIP, mercury intrusion porosimetry; PBS, phosphate buffered saline; PCL, poly(ε-caprolactone); PLGA, poly(lactic-co-glycolic acid); SAL-6, sterility assurance level of $10^{-6}$; $scCO_2$, supercritical carbon dioxide; SEM, scanning electron microscopy; Tg, glass transition temperature; THF, tetrahydrofuran; Tm, melting temperature; TSA, trypticase soy agar; TSB, trypticase soy broth.

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$10^{-6}$, *i.e.* one in a million. Bacterial endospores are non-reproductive forms of resistance of particular vegetative bacteria, protected with a thick protein coat to ensure their survival under harsh environmental conditions (e.g., high cell densities, extreme temperatures, drying, deprivation of nutrients, presence of chemical agents). This protection makes them virtually ubiquitous and represents a quality and health concern in the pharmaceutical and food industries (Checinska et al., 2015).

There is not a universal sterilization procedure suitable for the processing of all types of medical devices, including scaffolds, or biological tissues. This scenario represents a major hurdle in the development and commercialization of new generation biomedical products. Conventional sterilization treatments (steam/heat, ethylene oxide –EtO– and gamma radiation sterilization) are not suitable methods for most polymeric medical devices due to harsh experimental conditions, leading to the onset of physicochemical changes (Dai et al., 2016). Heat treatments are incompatible with the processing of most biodegradable polymers with low glass transition temperatures (Tipnis and Burgess, 2018). EtO treatment affects both the structural and biochemical properties of the treated scaffolds (Horakova et al., 2018; Zhao et al., 2019) and may lead to notorious changes in the drug release profiles of drug-loaded scaffolds (Hsiao et al., 2012). EtO residues or by-products are carcinogenic and mutagenic and specific limits of residual traces have been established for medical devices (Association for the Advancement of Medical Instrumentation et al., 2008), requiring tedious post-processing aeration steps (Shintani, 2017). Meanwhile $\gamma$-radiation is simple, rapid and effective against endospores, but it induces significant chemical changes in the polymeric scaffolds, and may affect the mechanical performance due to a decrease in the polymer molecular weight, thus varying the degradation rates after sterilization (Dai et al., 2016; Tipnis and Burgess, 2018).

Supercritical $CO_2$, $scCO_2$, is gaining increasing attention as a sterilization agent able to inactivate vegetative forms of a wide range of Gram-positive and Gram-negative bacteria (Ribeiro et al., 2019). The successful inactivation of bacterial endospores has been reported with the incorporation of low contents of additives (e.g. hydrogen peroxide, ethanol, peracetic acid, and mixtures of thereof) (Dai et al., 2016; White et al., 2006). The mild operating conditions, the low content of additives required and the excellent $scCO_2$ permeability are clear advantages for the sterilization of porous biomaterials for regenerative medicine purposes while maintaining the initial physicochemical properties of the material. Particularly, the effective sterilization of starch-based aerogels (SAL-6), the paradigm of labile nano-structured biomaterials, was achieved with minor modifications on the resulting textural properties and not compromising their mechanical performance (Santos-Rosales et al., 2019).

Polyesters, namely poly($\varepsilon$-caprolactone) (PCL) and poly(lactic-co-glycolic acid) (PLGA), are widely used to prepare biodegradable scaffolds (Deng et al., 2020; Feng et al., 2020; Go et al., 2020; Hedayati et al., 2020; Seddighian et al., 2021) and products from these materials are commercially available (Rapidsorb®, Osteomesh®, Osteoplug®). Their physical and mechanical properties and the degradation rate depend on the monomer ratio, the molecular weight and the crystallinity degree (Makadia and Siegel, 2011). These features allow the manufacture of scaffolds with specific degradation kinetics and mechanical properties, matching those of the targeted damaged tissue. For instance, PLGA of low inherent viscosity (0.2 dL/g) is particularly interesting for bone regeneration, fitting the 8 to 10 weeks degradation gap but its weak mechanical properties restrict its use (Diaz-Gomez et al., 2016). On the other hand, PCL presents higher strength and resilience, which can be advantageous for those tissues exposed to moderate mechanical stress, such as tendon, cartilage and bone, but shows slow degradation rate (complete PCL degradation in over 24 months) (Dash and Konkimalla, 2012). The similarity of the mechanical properties of PCL to those of trabecular bone has prompted the development of several grafts made of PCL solely or in combination with other polymers (Woodruff and Hutmacher, 2010).

The plasticizing effect of $scCO_2$ is exploited in the sc-foaming technology for the production of solvent-free PCL-based scaffolds, being compatible with the incorporation of thermosensitive compounds of interest, such as growth factors or active pharmaceutical ingredients (APIs) with yields close to 100% (García-González et al., 2015a; Santos-Rosales et al., 2020b). The scaffold structure (porosity, pore size distribution, mean pore size, pore interconnectivity) can be precisely controlled by modifications on the working parameters (temperature, pressure, soaking time and depressurization time) and the use of porogens (Chen et al., 2019; Salerno et al., 2017; Santos-Rosales et al., 2020a; White et al., 2012). However, the $scCO_2$ sterilization of preformed polyester-based scaffolds (PCL, PLGA) is not a valid post-processing step because the plasticizing effect of the $CO_2$ at the sterilization conditions may notably alter the internal and external architecture of the medical device. These processing limitations of $scCO_2$ technology can be overcome if sterilization conditions compatible with scaffolds production by supercritical foaming are identified and both processes are integrated. Thus, the aim of this work was to implement for the first time a $scCO_2$-based technological platform to solve the challenge of an integrated manufacturing of sterile biopolymer-based scaffolds (Fig. 1). $H_2O_2$ was used as additive that reinforces the sterilization capability of $scCO_2$ processing (Shieh et al., 2009). The sterilization conditions (pressure, temperature, time and $CO_2$ flow) were established after screening against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) vegetative bacteria and spores of *Bacillus stearothermophilus, Bacillus pumilus* and *Bacillus atrophaeus*. The effect of this two-in-one $scCO_2$ processing in PCL/PLGA scaffolds was evaluated in terms of physicochemical, morphological and mechanical properties. The outcomes were compared to those of the same scaffolds that underwent a conventional radiation sterilization (15 kGy). Residual levels of $H_2O_2$ were indirectly measured by incubation of the scaffolds with fibroblasts, a particularly sensitive cell line towards this additive (Shatrova et al., 2016).

## 2. Materials and methods

### 2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA) (50:50 lactic:glycolic ratio; 16 kDa; $T_g$ = 41.4 °C, amorphous) was purchased from Purac (Gorinchem, The Netherlands). Poly($\varepsilon$-caprolactone) (PCL) in the powdered form (50 kDa, $T_m$ = 61.4 °C, 66.7% crystallinity) was supplied by Polysciences (Warrington, PA, USA). $CO_2$ (purity of >99.9%) was provided by Praxair (Madrid, Spain). Trypticase soy broth (TSB) and trypticase soy agar (TSA) media were purchased from BIOKAR Diagnosis (Pantin, France)

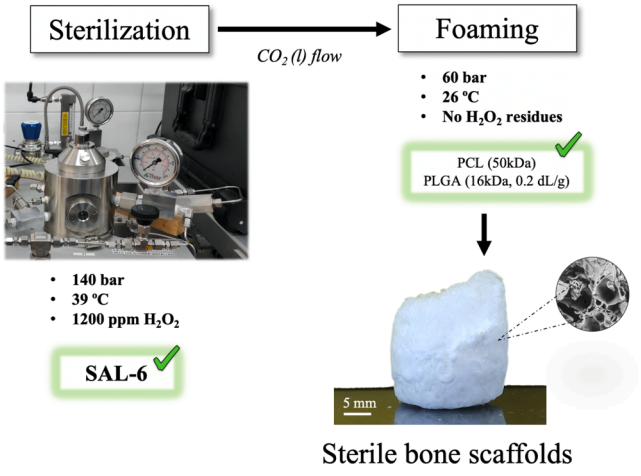


**Fig. 1.** Scheme of the developed supercritical $CO_2$-based technological platform for the manufacture of sterile polymeric bone scaffolds.

and hydrogen peroxide 30% (v/v) from Sigma-Aldrich (Madrid, Spain). Water was purified using reverse osmosis (resistivity > 18 MΩ·cm, MilliQ, Millipore, Madrid, Spain). Sterilization reel was purchased from E-line S.r.l. (Torre Pallavicina, Italy).

### 2.2. Development of the supercritical sterilization method

#### 2.2.1. Efficacy of the $scCO_2$ sterilization treatment against vegetative bacteria

300 mL of TSB liquid medium with an initial concentration of $10^8$ CFU/mL were prepared from both Gram-positive (*Staphylococcus aureus* -ATCC 25923-) and Gram-negative vegetative bacteria (*Escherichia coli* -ATCC 25922-; *Pseudomonas aeruginosa* -CECT 110-) in HPLC-vials previously sterilized with a perforated cap to allow the $CO_2$ diffusion. Vials were placed in triplicate into thermally sealed sterilization pouches. Afterwards, the vials were located in a stainless-steel autoclave of 600 mL (NovaGenesis, NovaSterilis Inc., Ithaca, NY, USA) with the respective amount of additive (600 ppm of $H_2O_2$). The system was heated to 37 °C and pressurized at a constant rate of 20 g/min until 140 bar. Depending on the trial (Table 1), the setup was maintained in the batch mode for 1 to 4.5 h and depressurized (2–4 bar/min) until reaching atmospheric pressure. Once the system was depressurized, the vials were collected and 1 mL of each vial was plated onto TSA medium to quantify the reduction of the initial bioburden. After 24 h of incubation at the optimal temperature (37 °C), the counting of the microbial colony growth (CFUs/mL) was performed.

#### 2.2.2. Efficacy of the $scCO_2$ sterilization treatment against spores

Commercial spore strips with $10^6$ spores of *Bacillus stearothermophilus* (ATCC 7953) and *Bacillus pumilus* (ATCC 27142) were purchased from Sigma-Aldrich, Inc. (Madrid, Spain). *Bacillus atrophaeus* (cell line 9372) spores were obtained from Crosstex International, Inc. (Rush, NY, USA). These three microorganisms are the biologicals indicators used in steam and hydrogen peroxide vapor sterilizations (ISO 17665–1:2006, 2006), γ-radiation sterilization (ISO 11137–1:2006/Amd.1:2013, 2013) and ethylene oxide or dry heat sterilization (ISO 11135:2014, 2014), respectively. The spore strips were sealed and placed in a 100-mL stainless steel autoclave (Thar Process, Pittsburg, PA, USA) equipped with a top agitation system (700 rpm), including different amounts of $H_2O_2$ as additive (600 or 1200 ppm of $H_2O_2$). The autoclave was then heated to 39 °C and pressurized until 140 bar at a constant pressurization rate of 13.3 bar/min. Depending on the assay, the system was maintained in batch mode or combined with a continuous flow of $CO_2$ at 5 g/min. Finally, the system was depressurized at a constant venting rate of 3.2 bar/min until atmospheric pressure. The bacterial growth was evaluated by naked eye through turbidity tests after 7 and 14 days of incubation without stirring (Raypa Digital Incubators, Terrassa, Spain) of the strips in tubes containing 10 mL of TSB medium, under the corresponding recommended incubation temperatures (37 °C for *B. pumilus* and *B. atrophaeus*, and 60 °C for *B. stearothermophilus*). In addition, the absence of growth was confirmed by seeding 1 mL of the medium after 7 and 14 days in TSA plates.

**Table 1**
Supercritical sterilization trials of bacteria suspensions at 37 °C and 140 bar. The efficacy of the process was reported as logarithmic reduction of the initial microbial burden (logR).

| Microorganism | Initial microbial concentration | Time (h) | Additives | logR |
|---|---|---|---|---|
| *Staphylococcus aureus* | $10^8$ CFU/mL | 1 | 600 ppm $H_2O_2$ | 5 |
| | $10^8$ CFU/mL | 4.5 | 600 ppm $H_2O_2$ | 8 |
| *Escherichia coli* | $10^8$ CFU/mL | 1 | 600 ppm $H_2O_2$ | 8 |
| | $10^8$ CFU/mL | 4.5 | 600 ppm $H_2O_2$ | 8 |
| *Pseudomonas aeruginosa* | $10^8$ CFU/mL | 4.5 | 600 ppm $H_2O_2$ | 8 |

### 2.3. One-pot supercritical $CO_2$ sterilization and foaming

PCL and PLGA (1:1 wt ratio) in the powdered form were manually mixed using a spatula (1 g) in cylindrical Teflon molds (L: 24.6 mm, D: 17 mm) (Brand GmbH, Wertheim, Germany), and then compacted with an aluminum plunger. The filled molds were then placed in the same supercritical equipment used in the spore sterilization assays (*see Section 2.2.2)* and subjected to sterilization conditions (test #6, Table 2). The transition from the sterilization conditions (140 bar, 39 °C) to the compressed foaming parameters (60 bar, 26 °C), was performed through a controlled depressurization (3.2 bar/min) followed by a flow (20 g/min) of liquid at 4 °C during 15 min to cool down the vessel to 26 °C (Garcia-Gonzalez et al., 2015b). The system was maintained at 60 bar and 26 °C for 1 h. Afterwards, the pressure was reduced to 38 bar at a constant flow rate of 20 bar/min, then by the addition of liquid $CO_2$ the pressure was raised again to 60 bar. These depressurization/pressurization cycles were performed 3 times. Lastly, the system was depressurized to ambient pressure at a venting rate of 20 bar/min. A magnetic stirrer was placed at the bottom of the autoclave (not in contact with samples) and maintained constant at 700 rpm during the process. A general scheme of the pressure profile of the method is depicted in Fig. 2. Scaffolds in the form of solid porous cylinders were collected from the autoclave, labeled and stored for further characterization.

### 2.4. γ-sterilization of scaffolds

To compare the effects of the $scCO_2$ sterilization treatment, scaffolds manufactured according the same procedure as in Section 2.3 but in absence of the $H_2O_2$ additive (unsterile) were packed in individual containers, and then they were γ-irradiated (Ob-Servo Ignis 06, Institute of Isotopes Co., Ltd. (IZOTOP), Budapest, Hungary) at 4.8 kGy/h using a $^{60}Co$ source to reach a total dose of 15 kGy.

### 2.5. Scaffold characterization

#### 2.5.1. Structural and physicochemical characterization

Micrographs of the scaffolds were obtained by scanning electron microscopy (FESEM, ULTRA PLUS, Zeiss, Oberkochen, Germany).

Bulk density values ($\rho_{bulk}$) were calculated from the dimensions and weight of the manufactured scaffolds. The skeletal density ($\rho_{skel}$) was determined using a helium-pycnometer (Quantachrome, Boynton Beach, FL, USA) at room temperature (25 °C) and 1.01 bar (six replicates). Overall porosity ($\varepsilon$) was calculated following Eq. (1).

$$\varepsilon(\%) = \left(1 - \frac{\rho_{bulk}}{\rho_{skel}}\right) \times 100 \quad (1)$$

Relative molecular mass data of polymer samples were obtained by gel-permeation chromatography (GPC) using a differential refractive-index detector (Waters Model 410, USA) with serial Styragel columns from Waters (HR 6, HR 4, HR 3, HR 1 and HR 0.5), which covered from 0 to $1.0{\cdot}10^7$ Dalton of effective molecular weight, as specified by the manufacturer. The analysis was done at 40 °C using tetrahydrofuran (THF) at a flow rate of 1 mL/min as mobile phase and a sensibility of 32. A 100-μL sample of each solution was injected into the system and data collection and analysis were performed using Breeze software® (Waters, Milford, MA, USA).

Nine polystyrene standards with low polydispersity (2.8–$8.6{\cdot}10^6$ KDa, Toyo Soda, Japan) were used to elaborate a third calibration curve (Log Mol Wt = $145–11.6{\cdot}V+0.319{\cdot}V^2–0.00297{\cdot}V^3$; $R^2 = 0.976$; standard error = 0.217).

Polymer samples of 0.8 mg/mL were prepared by direct dissolution in the mobile phase at 40 °C with continuous magnetic stirring and

**Table 2**
$ScCO_2$ sterilization tests against bacterial endospores at 39 °C and 140 bar and the achieved inactivation degree. Notation: *Y* denotes that SAL-6 levels were reached.

| Test | Operating mode | Time[a] (h) | $H_2O_2$ content (ppm) | SAL-6 for *B. stearothermophilus* | SAL-6 for *B. pumilus* | SAL-6 for *B. atrophaeus* |
|---|---|---|---|---|---|---|
| #1 | Static | 2.5 | 600 | – | – | *Y* |
| #2 | Static | 5 | 600 | *Y* | – | *Y* |
| #3 | Static | 5 | 1200 | *Y* | *Y* | *Y* |
| #4 | Static | 2.5 | 1200 | *Y* | *Y* | *Y* |
| #5 | Combined | 5 (2.5 + 2.5) | 1200 | *Y* | *Y* | *Y* |
| #6 | Combined | 3 (2 + 1) | 1200 | *Y* | *Y* | *Y* |

[a] In the parenthesis, the first term is the time maintained in static mode and the second term is the time under $CO_2$ flow.

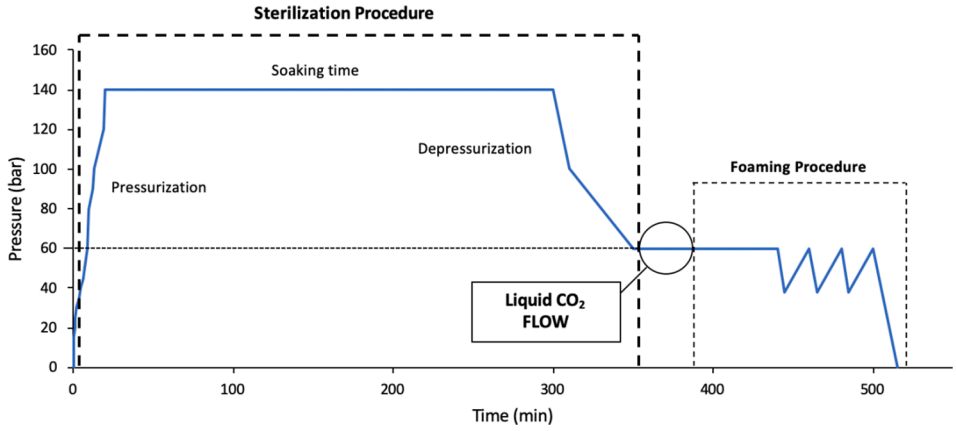


**Fig. 2.** Scheme of the experimental procedure to integrate the sterilization of the material (first part) and the controlled foaming (second part).

analyzed during the same day. Relative molecular mass of polymer samples was calculated based on the calibration curve using the abovementioned software. To calibrate the system and monitor its performance, a polystyrene standard of $10.2{\cdot}10^4$ Da was analyzed daily.

### 2.5.2. Mercury intrusion porosimetry (MIP) analyses

MIP analyses of the scaffolds were performed (Autopore IV 9500 model, Micromeritics, Norcross, GA, USA) at working pressures ranging from 0.07 to 1800 bar to determine their pore size distributions. Porosity values ($\varepsilon_{MIP}$) were determined from the intruded volume of mercury ($V_{p_{MIP}}$) in the scaffolds with the increase of pressure using the Washburn equation (Ho and Hutmacher, 2006).

### 2.5.3. Mechanical properties

Scaffolds (in duplicate) were subjected to unidirectional cyclic compression tests (five times) in a tensile bench with a 30 kg load cell (TA.XT*Plus*, Stable Micro Systems, Ltd., Godalming, UK) at a crosshead speed of 1 mm/min. Each compression cycle was performed up to a maximum strain of *ca.* 20%. All the experiments were performed at room temperature (25 °C), atmospheric pressure and 45% relative humidity. Elastic deformation was expressed in percentage and calculated from the ratio between the initial height and the height of the sample bearing the highest applied physical stress. The Youngs modulus (E) was calculated from the stress–strain plots previous conversion to engineering stress and engineering strain.

## 2.6. Cytotoxicity tests

Murine fibroblast (CCL-163, ATCC, USA) were employed to evaluate the cytotoxicity of the treated scaffolds, according to ISO 10993–5:2009 standard (ISO 10993–5:2009, 2009). $H_2O_2$ is highly toxic to fibroblast and its presence in a residual content absorbed on the $scCO_2$-PCL/PLGA scaffolds was evaluated using two different strategies.

In one method, scaffolds were put in contact with cells and cytotoxicity was evaluated at 24 and 72 h. Briefly, cells were seeded in 24-well plates (20,000 cells/well) with 0.5 mL of culture medium of DMEM supplemented with 10% FBS and 1% penicillin (10,000 UI/mL)/streptomycin (10,000$\mu$ g/mL). Cells were maintained at 37 °C in a humidified atmosphere enriched with 5% $CO_2$ for 6 h to allow their attachment to the bottom of the well. Cubic scaffold pieces of 50 mg were incubated with cells in quintuplicate for 72 h at the former conditions. Controls included cells incubated without material (negative control) and cells incubated with PCL/PLGA scaffolds sterilized by soaking in EtOH 70% for few minutes, followed by PBS washes (positive control).

The second method involved the incubation of the scaffolds with the complete medium to allow the diffusion of any $H_2O_2$ residues trapped in the polymeric matrix. Scaffold pieces (50 mg; n = 5) were put in 24-well plates with 1 mL of culture medium and incubated for 72 h at 37 °C in a humidified atmosphere with 5% $CO_2$. Eluates of 200 $\mu$L were removed at 24, 48 and 72 h and seeded in 96-well plates containing the fibroblast (30,000 cells/cm$^2$). The removed supplemented medium was not replaced to avoid a dilution effect of the cytotoxic potential of $H_2O_2$ residues. Controls included cells incubated with eluates from PCL/PLGA scaffolds sterilized by soaking in EtOH (positive control) and culture medium (negative control).

Cell proliferation was evaluated using the cell counting kit-8 (CCK-8) (Roche, Switzerland) at the sampling times and performed according to the manufacturer protocol. Absorbance was read at the wavelength of 450 nm (UV BioRad Model 680 microplate reader, Hercules, CA, USA). Experiments were carried out in quintuplicate and the cell viability (%) calculated as follows:

$$Cell\ \ viability\ \ (\%) = \frac{Abs_{exp}}{Abs_{negative\ \ control}} \times 100 \qquad (2)$$

## 2.7. Statistical analysis

All results were expressed as mean ± standard deviation. Statistical analyses of the morphological, mechanical and cytocompatibility results (1-way ANOVA) were performed using Statistica v8.0 software (StatSoft Inc., Tulsa, OK, USA) followed by the post hoc Tukey HSD multiple

comparison test.

## 3. Results and discussion

### 3.1. Screening of operating conditions for inactivation of vegetative and endospore bacterial forms

The one-pot sterilization and foaming of polymer scaffolds required first the identification of sterilization conditions that can provide SAL-6 while still being compatible with the polymer processing. The sterilizing effect of the $scCO_2$ relies on a combination of multiple mechanisms of varying relevance in the inactivation of microorganisms. Among them, the acidification of cytoplasm and further damage of the cell envelope may play a major role in the inactivation mechanism of $scCO_2$, which are favored by $CO_2$ solubilization in the aqueous medium containing the bacteria (Ribeiro et al., 2019). $CO_2$ is also able to extract lipids from the cell envelope (Kamihira et al., 1987; Sahena et al., 2009). $scCO_2$ acts as a sterilizing agent by itself, but also as a solvent that enhances the biocidal function of additives like $H_2O_2$ in the inner parts of the sterilized materials (Hâncu et al., 2002).

Initial sterilization tests were performed on vegetative forms of both Gram-positive (*S. aureus)* and Gram-negative (*E. coli*, *P. aeruginosa)* bacteria with $scCO_2$ and in the absence of additives. The pathogens were selected regarding their high incidence as etiological agents of medical device-associated infections once implanted (Johnson and García, 2015). Mild temperature (37 °C) and moderate pressure (140 bar) for short soaking times (1 h) were enough to achieve a complete reduction (SAL-8) on the suspensions containing *E. coli* without the presence of additives. However, the reduction achieved for *S. aureus* was only of SAL-5 under these conditions. These results are related to the fact that the bacterial cell walls (in terms of composition and thickness) of Gram-positive and Gram-negative bacteria are different. For instance, the peptidoglycan layer is much thinner in Gram-negative species, which facilitates the $scCO_2$ diffusion, and thus accelerates the acidification of the cell cytoplasm (Zhang et al., 2006c). In this context, Gram-negative bacteria were more easily inactivated with the $CO_2$ treatment (Table 1).

The incorporation of low $H_2O_2$ contents (600 ppm; Table 1) enhanced the sterilization efficacy against *S. aureus*, but the obtained bioburden reductions were similar to pure $scCO_2$. This effect can be attributed to the water contained in the liquid TSB, which can be indirectly acting as a sterilization additive.

The contact time with $scCO_2$ was identified as the crucial factor influencing the antimicrobial effect, and 4.5 h were required for the complete inactivation of *S. aureus* under 600 ppm of $H_2O_2$. Under these conditions, the complete inactivation of vegetative forms of *P. aeruginosa* was also successfully achieved (SAL-8) (Fig. 3).

Conventional sterilization methods are validated against a biological indicator, which is the bacterial spore strip more resistant to the procedure. To date, there is not an international standard to be used as biological indicator of $scCO_2$ sterilization, encouraging the use of those from the standard sterilization procedures (steam, radiation and ethylene oxide) (Table 2), where the absence of bacterial growth implied a reduction of $10^6$ (SAL-6). The efficacy of the supercritical sterilization treatment was thus assessed against the three commercial bioindicators.

The use of agitation during the supercritical trials was needed for the full inactivation of endospores. Agitation ensured a homogeneous distribution of $H_2O_2$ within the autoclave. An increase of 2 °C in the working temperature was required to inactivate bacterial endospores under 140 bar.

*B. atrophaeus* spores were the most sensitive towards the $scCO_2$ treatment among the tested biological indicators. Under low $H_2O_2$ concentrations (600 ppm), less than 3 h were required to achieve a complete inactivation of *B. atrophaeus* spores (test #1, Table 2). Differently, the other bioindicators grew in the first 24 h post-treatment. The resistance of *B. atrophaeus* towards $scCO_2$ sterilization in presence of $H_2O_2$ was already reported to be lower than that exhibited by other *Bacillus* genus species (Zhang et al., 2006a, 2006b). At the same additive content (600 ppm), longer soaking times (5 h) were enough to accomplish a SAL-6 for *B. stearothermophilus* spores (test #2, Table 2). Finally, higher additive contents (1200 ppm) were required to reach SAL-6 for *B. pumilus* spores (test #3, Table 2). Lower exposure time (2.5 h) also caused total inactivation of the three spore strains (test #4, Table 2). Since the resistance of *B. pumilus* spores was superior to the rest of the tested biologicals indicators, *B. pumilus* strain is proposed as the biological indicator of reference to evaluate the efficacy of sc-sterilization treatment.

Moreover, remnants of liquid $H_2O_2$ were detected when the autoclave was opened after static sterilization tests (tests #1–4, Table 2).

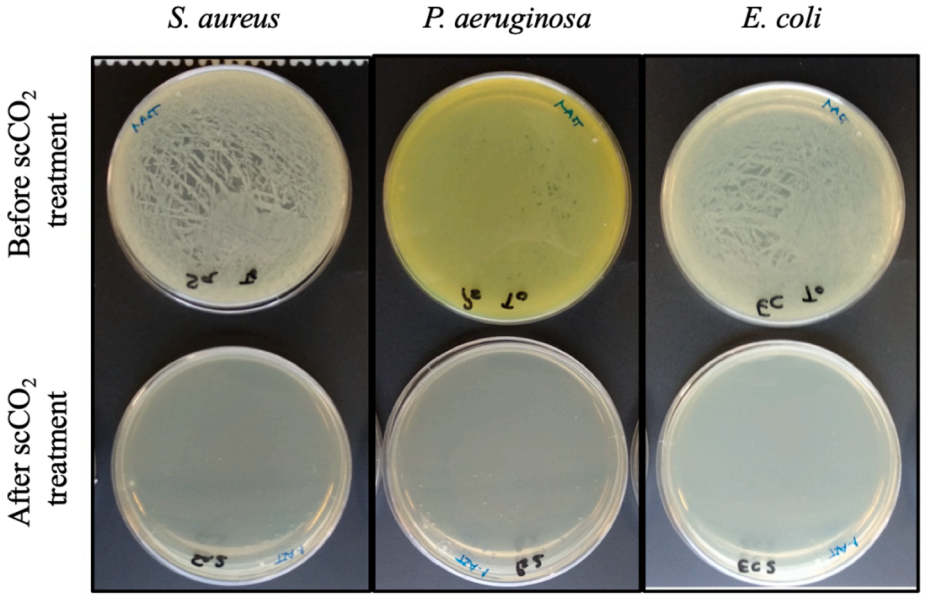


**Fig. 3.** TSA plates highlighting the complete reduction (bottom pictures) of the initial microbial burden of *S. aureus*, *P. aeruginosa* and *E. coli* after the supercritical $CO_2$ sterilization at 37 °C and 140 bar in the presence of 600 ppm of $H_2O_2$ as additive. For the sake of comparison, the bacterial mantle formed when $10^8$ CFUs/mL are seeded in the plate is provided (top pictures).

Traces of $H_2O_2$ in the sterilized materials can cause patient discomfort or even toxicity (Mahaseth and Kuzminov, 2017; Nichols et al., 2019); thus, post-processing steps (e.g. aeration) are usually required when treating biomaterials (Gülden et al., 2010). To solve this drawback, a dynamic process consisting on the combination of a batch mode with subsequent $CO_2$ flow was implemented. Under these conditions, SAL-6 was achieved against the biological indicators and no residual $H_2O_2$ was detected within the autoclave (tests #5 and #6, Table 2).

Overall, the sterilization efficacy of the presented $scCO_2$-based methods relies on the synergistic effect of $CO_2$ and the additive. A significant softening of the working parameters (temperature, pressure and soaking time) to achieve SAL-6 against dry spores with respect to the literature (Donati et al., 2012; Shieh et al., 2009; Zhang et al., 2006a, 2006b) has been achieved. In addition, the absence of additive at the end of the sterilization procedure may pave the way towards its implementation for routine biomaterial sterilization. The optimum experimental conditions in terms of sterilization efficacy, economy of the process, and absence of residual $H_2O_2$ in the autoclave were identified as those corresponding to test #6 (Table 2).

### 3.2. Sterilization and foaming integration process

The optimized $scCO_2$ sterilization method (test #6, Table 2) was adapted for the production of sterile PLGA/PCL foamed scaffolds in a one-pot integrated process (*see section 2.3)*. This approach was developed for the foaming of polymers of low inherent viscosity (0.2 dL/g), which is still technologically challenging (Diaz-Gomez et al., 2016). The transition from the sterilization parameters of pressure and temperature to those of foaming was performed by a controlled depressurization and a fast cooling of the autoclave, respectively. The depressurization step of the foaming process strongly determines the foam formation (Di Maio and Kiran, 2018), and the polymer mixture used in this work may suffer an unconstrained pore expansion leading to a loss of physical integrity if the depressurization is not precisely controlled. Firstly, the pressure was reduced up to 60 bar, since below this threshold the polymer expansion was uncontrolled, according to preliminary foaming results (data not shown). The temperature transition from 39 to 26 °C was critical to control the polymer matrix expansion, and the use of a liquid $CO_2$ flow resulted in a fast and efficient approach to cool down the autoclave. After the cooling step, the system was maintained for 1 h at 60 bar to reach stable $CO_2$ sorption levels in the polymeric mixture. To force the polymer vitrification and restrict the foam expansion, depressurization/pressurization cycles were performed during the depressurization step to further cool down the autoclave, as previously reported (Goimil et al., 2018).

Following the proposed procedure, the polymer expansion was precisely controlled and sterile lightweight foams were manufactured ($scCO_2$-PCL/PLGA in Table 3). Overall, homogeneous and highly porous scaffolds (>71%) were obtained (Fig. 4), matching those of trabecular bone (Dwivedi et al., 2020; Wang et al., 2020). SEM images unveiled the presence of a family of interconnected large (>100 μm) pores surrounded by smaller pores. Although the optimal pore size in scaffolds for long bone regeneration is above 300 μm (Abbasi et al., 2020; Lee et al., 2019), smaller pores have been demonstrated to play a beneficial role in the mechanical performance, ultimately leading to favorable *in vivo* tissue repair in critical calvarial defects using a rat model (Wang et al., 2020). The porosity determined by mercury intrusion porosimetry ($\varepsilon_{MIP}$) was clearly different to the overall porosity values, with a rough decrease of 50% (Table 3). By the MIP-method performed in this work, only open pore populations in the 0.01–180 μm range were measured. The presence of closed pores or pores larger than the upper limit (>180 μm) were responsible for these divergences.

### 3.3. Effect of the sterilization technique on the scaffold properties

The influence of the sterilization techniques on the structural properties of the manufactured scaffolds was evaluated. Particularly, $scCO_2$ sterilization was compared to a standard technique ($\gamma$-irradiation). In addition, the following controls were produced and evaluated: unsterile scaffolds (negative controls) were manufactured applying the sterilization/foaming integrating process but in absence of $H_2O_2$ (PCL/PLGA, in Table 3). Some of these scaffolds were further subjected to a standard gamma radiation method of 15 kGy (Nguyen et al., 2011) and considered as positive controls ($\gamma$-PCL/PLGA, in Table 3).

Scaffolds subjected to the γ-radiation treatment experimented a significant densification ($p < 0.05$), reducing the overall porosity values by *ca.* 10% with respect to unsterile scaffolds. The smaller pore population (<180 $\mu$m) was particularly affected by this densification, since the decrease in the $\varepsilon_{MIP}$ directly correlates with the overall porosity reduction (Table 3). This effect can restrict the use of γ-sterilization procedures for polymeric scaffolds, since small pores play a major role in increasing the surface area and enhancing cell colonization (Jodati et al., 2020).

Irradiated scaffolds underwent a remarkable decrease in $M_w$ and $M_n$ (GPC data in Table 3), indicating that the $\gamma$-sterilization procedure degraded the polymers shortening their chains (Cottam et al., 2009; Holy et al., 2000). The significant reduction on the molecular weight observed for the γ-irradiated scaffolds was mainly attributed to the PLGA degradation, since decreases of 50–70% were previously reported for this biopolymer depending on the irradiated dose (Holy et al., 2000; Shahabi et al., 2014). These significant changes in the polymer at the irradiated dose (15 kGy) discouraged the gamma irradiation for the sterilization of the manufactured scaffolds. It should be noted that the tested irradiation dose was already below the standard value (25 kGy). Thus, although lower doses could preserve the physicochemical properties of the scaffolds, their sterility might be jeopardized (Bosworth et al., 2012).

Remarkably, when the $scCO_2$ sterilization/foaming procedure was applied, the resulting scaffolds were morphologically identical to their unsterile counterparts (Table 3). The $H_2O_2$ addition slightly favored the expansion of the foams during the manufacturing, although no statistically significant differences in densities were detected compared to the untreated material. Supercritical sterilization had no impact in the chemical properties of the polymeric mixture since identical molecular weights ($M_w$, $M_n$) to the unsterile scaffolds were obtained by GPC analysis (Table 3).

Mechanical evaluations showed that the scaffolds maintained their physical integrity and cracks were not formed during the compression test. Scaffolds had an elastoplastic behavior during the compression cycles with permanent deformation (Fig. S1). The structures became stiffer after several uniaxial stress cycles with a 20% increase in the Young's moduli after 5 cycles (Table 4) and being suitable for the intended application (Athanasiou et al., 2000). No statistical differences were observed in the mechanical properties between scaffolds,

**Table 3**
Physicochemical properties of PCL/PLGA scaffolds subjected to sterilization treatments (γ-sterilization; $scCO_2$ sterilization) compared to untreated scaffolds. Equal letters denote statistically homogeneous groups (1-way ANOVA, $p < 0.05$, and post hoc Tukey HSD multiple comparison test).

| | $\rho_{bulk}$ (g/cm$^3$) | $\rho_{skel}$ (g/cm$^3$) | $\varepsilon$ (%) | $\varepsilon_{MIP}$(%) | $V_P$ (cm$^3$/g) | $M_w$ (kDa) | $M_n$ (kDa) | PD |
|---|---|---|---|---|---|---|---|---|
| PCL/PLGA | $0.308 \pm 0.018^a$ | $0.998 \pm 0.008$ | $69.1 \pm 1.8^a$ | 31.1 | 2.25 | 80.9 | 18.0 | 4.5 |
| $\gamma$-PCL/PLGA | $0.390 \pm 0.023^b$ | $0.974 \pm 0.004$ | $60.0 \pm 2.4^b$ | 23.2 | 1.54 | 27.8 | 12.2 | 2.3 |
| $scCO_2$-PCL/PLGA | $0.289 \pm 0.011^a$ | $1.000 \pm 0.012$ | $71.1 \pm 1.2^a$ | 30.0 | 2.46 | 81.8 | 21.5 | 3.8 |

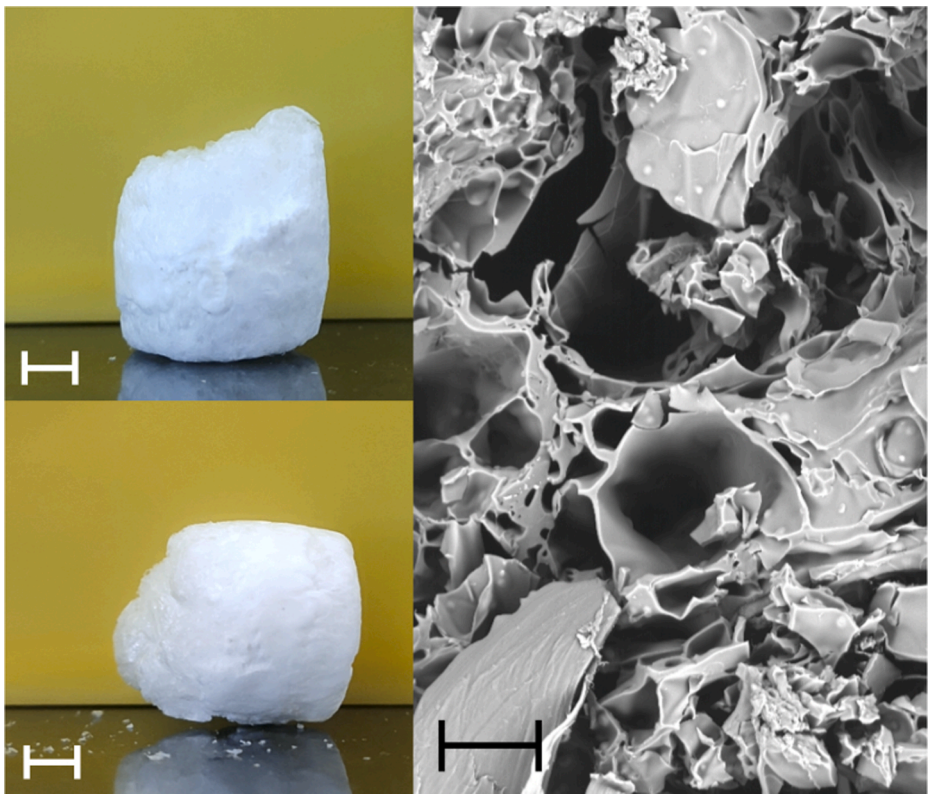

**Fig. 4.** Images of PCL/PLGA scaffolds produced by the one-pot sterilization/foaming method. Scale bars: 5 mm (white) and 100$\mu$ m (black).

**Table 4**
Compressive mechanical properties of PCL/PLGA scaffolds after being subjected to the sterilization treatments ($\gamma$-sterilization; $scCO_2$-sterilization) compared to untreated scaffolds.

| Scaffold | Compression Cycle | Young's Modulus (MPa) |
|---|---|---|
| PCL/PLGA | 1 | 6.73 ± 0.46 |
| | 5 | 7.32 ± 0.34 |
| $\gamma$-PCL/PLGA | 1 | 6.29 ± 0.50 |
| | 5 | 7.09 ± 0.36 |
| $scCO_2$-PCL/PLGA | 1 | 6.35 ± 0.24 |
| | 5 | 7.91 ± 0.61 |

regardless of the sterilization treatment performed.

Despite the reduction on the molecular weight of γ-irradiated scaffolds, the mechanical behavior was unaltered compared to untreated foams. The reduction on the molecular weight of PLGA upon γ-irradiation did also not show any effect on the mechanical performance of solely PLGA scaffolds (Davison et al., 2018). On the other hand, a standard γ-irradiation procedure (25 kGy) on PCL scaffolds was reported to induce an increase in molecular weight due to cross-linking of polymer chains, ultimately increasing the yield stress (Cottam et al., 2009). These effects of radiation on the chemical structure of the polymers may counteract any change in the mechanical performance.

### 3.4. Cytotoxicity tests of $scCO_2$ sterilized/foamed PCL/PLGA scaffolds

The presence of residual $H_2O_2$ absorbed in the sterile PCL/PLGA scaffolds produced under the most adequate conditions (test #6, Table 2) was indirectly assessed using fibroblasts. Cytotoxicity derived from $H_2O_2$ concentrations in cell culture can strongly vary with the cell line, incubation time and cell density (Gülden et al., 2010). Intracellular $H_2O_2$ concentrations above 1 μM are known to induce an oxidative stress to the cells, ultimately leading to death (Stone and Yang, 2006). The proposed dynamic step with $CO_2$ flow during the sterilization/foaming procedure reduced the presence of the additive on the individually packed sterile scaffolds up to non-toxic levels.

$scCO_2$-PCL/PLGA scaffolds exhibited outstanding viability values, regardless of the experimental method used (Fig. 5). A slightly yellowish coloration on the medium containing the scaffolds was observed and attributed to an acidification due to PLGA degradation. Nevertheless, cells directly incubated with scaffold pieces presented a good morphology with cell viability values close to those obtained for the controls. Cells incubated for 24 h in the presence of $scCO_2$-PCL/PLGA scaffolds had the lowest viability values ($p < 0.05$), although the viability was still above 80%. After 72 h of direct contact of scaffolds with fibroblasts, there were no significant differences in the cell viability values either from controls or EtOH sterilized scaffolds (Fig. 5A). Cells incubated with the scaffolds eluates also presented an appropriate shape and grew in the same extent or even more than the controls along the three tested time periods (24, 48 and 72 h) (Fig. 5B).

## 4. Conclusions

An integrated sterilization/foaming procedure for scaffold manufacturing based on the use of $scCO_2$ technology is herein firstly reported. Sterile PCL/PLGA scaffolds were obtained, meeting the morphological and mechanical criteria to be used as bone graft substitutes. The method allowed for an excellent morphological control and preserved the chemical identity of foams containing PLGA of low inherent viscosity (0.2 dL/g), a technologically challenging polymer. Despite the $H_2O_2$ addition and absence of post-processing aeration steps, no cytotoxicity was observed in the resulting scaffolds once incubated with fibroblasts, a particularly sensitive cell line versus this additive. Differently, the gamma sterilization procedure (15 kGy) led to significant structural and physicochemical changes, compromising the scaffold quality. Results place great value on the supercritical fluid technology as a dual (sterilizing + foaming) tool to address critical challenges of tissue engineering: sterilization of biomaterials and scaffold process

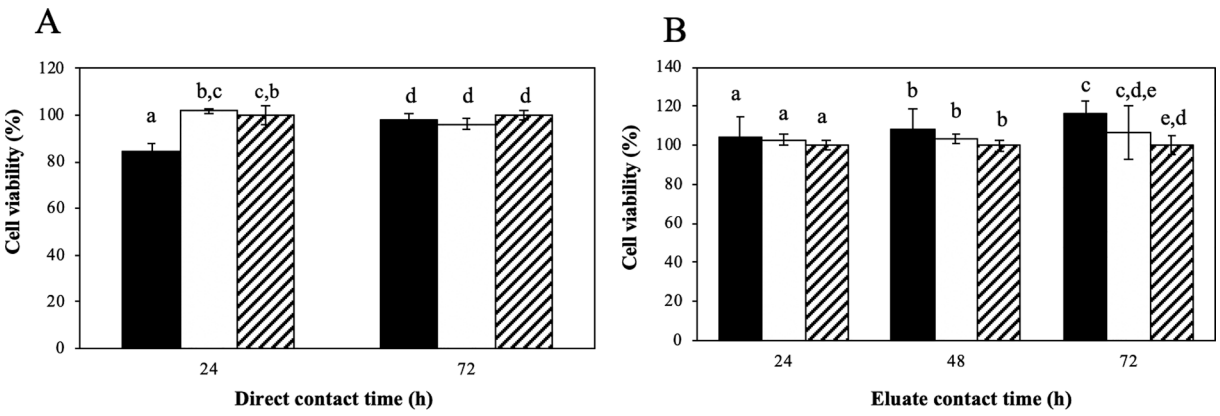


**Fig. 5.** Cell viability studies determined by WST-8 test after 24 and 72 h in direct contact (A) and after 24, 48 and 72 h in contact with eluates (B) of manufactured PCL/PLGA scaffolds. Legend: $scCO_2$ sterilized scaffolds (black bars), EtOH sterilized scaffolds (white) and controls (striped). Equal letter denotes statically homogeneous groups (1-way ANOVA; $p < 0.05$).

integration. These translational results will be further tested in an *in vivo* animal model regarding bone repair. Finally, spores of the *B. pumilus* strain are proposed as the biological indicator of reference to assess $scCO_2$-based sterilization methods.

## CRediT authorship contribution statement

**Víctor Santos-Rosales:** Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Beatriz Magariños:** Conceptualization, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Ricardo Starbird:** Investigation, Writing - review & editing. **Javier Suárez-González:** Investigation, Writing - review & editing. **José B. Fariña:** Investigation, Writing - review & editing. **Carmen Alvarez-Lorenzo:** Funding acquisition, Writing - original draft, Writing - review & editing. **Carlos A. García-González:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120801.

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