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Sterilization of macroscopic poly(L-lactic acid) porous scaffolds with dense carbon dioxide: Investigation of the spatial penetration of the treatment and of its effect on the properties of the matrix

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ABSTRACT

In this work the sterilization with dense carbon dioxide of poly(L-lactic acid) (PLLA) porous scaffolds intended for tissue engineering applications was investigated with the main objective of confirming the three-dimensional efficacy of the treatment and of analysing the scaffold properties after CO_2 treatment. For this purpose the scaffold was contaminated with a conventional bacterium (*Escherichia coli*) and with spores (*Streptomyces coelicolor*), a species more fascinating and difficult to inactivate. Contamination was performed in such a way to soak the whole matrix with bacteria and spores. The effect of pressure and treatment time on the efficacy of the sterilization was evaluated. The *E. coli* was eradicated from the whole matrix after just 5 min of exposure to supercritical CO_2 at 10 MPa and 40 °C. The spores required more severe conditions due to their different and very resistant structures since complete inactivation was obtained after 360 min of exposure at 30 MPa and 40 °C or at 30 MPa and 30 °C after the addition of 200 ppm of H_2O_2 to near critical CO_2. The treatment with dense CO_2 did not alter the biocompatibility and the structure of the scaffold as demonstrated by biological culture tests and calorimetric and SEM analyses.

Collected data suggest that dense CO_2 is a promising alternative to conventional sterilization techniques to sterilize biodegradable PLLA scaffolds.

1. Introduction

The main objective of tissue engineering concerns the regeneration of damaged tissue or the creation of artificial organs [1]. Porous polymer matrixes were often used as supports to allow cell adhesion and proliferation and the nature of materials depends on the specific application [2]. For instance, synthetic resorbable polyesters such as poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(D,L-lactic acid) (PDLLA), poly(glycolic acid) (PGA) and poly(L-lactic glycolic acid) (PLGA) were already used in a large number of approved medical implants because they are considered safe, non-toxic and biocompatible [3]. The main challenge of these materials is related to their functionality and not only to biocompatibility. Indeed a biomaterial must be able to interact with tissues and exercise functions for which it was designed and implanted; in this context, very important properties of scaffolds are their pore size, architecture and interconnection degree [4]. Furthermore, these materials can be modified to improve cellular affinity and chemical versatility [5–7].

Surely, prior to the final application and utilization of these materials, it is necessary to provide their "total sterilization". The term "total" refers to the sterilization of both the external and internal surface of these complex porous structures. Before implantation, as described by Cholvin and Bayne [6], these matrixes can be submitted to two different processes: cleaning, that physically eliminates residues or particulates, and sterilization, that allows abatement of population of living species. Our interest is focused on the sterilization method defined also as a procedure to obtain a 6-log reduction in colony forming units (CFUs) of contaminants [8]. The most common methods used until now to

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sterilize biomedical devices are ethylene oxide exposure, steam sterilization, γ-irradiation and hydrogen peroxide plasma, but all of them exhibit some drawbacks [9]. The ethylene oxide can remain adsorbed in the material after the treatment and may cause toxicity and induce chemical reactions that damage the polymer structure [10,11]; steam sterilization cannot be used for thermolabile and hydrolytically sensitive polymers due to its high temperature [12]; γ-irradiation induces degradation of the polymeric matrixes by cross-linking or chain scission reactions leading to relevant changes in the molecular architecture of the materials [13]; finally, free radicals generated during the hydrogen peroxide plasma process may adversely react with the polymer chains of the sterilized materials and degrade them [14].

In this context, supercritical carbon dioxide ($scCO_2$) is an alternative solvent to use, with and without suitable additives, in order to create an adverse environment for the microorganism survival.

As reviewed earlier [8], the use of $scCO_2$ is an excellent alternative for several reasons: (i) $scCO_2$ is not flammable, nontoxic, chemically stable in the absence of basic compounds and hence it is not expected to react with polymers; (ii) it presents mild critical temperature (31.1 °C), which is only slightly above room temperature, so thermal degradation is usually not a problem when the process is operated around the critical temperature; (iii) at supercritical state CO_2 has low viscosity and being monophasic it exhibits no capillary forces in pores so it can quickly penetrate complex structures and porous materials; (iv) CO_2 is inexpensive and readily available, which makes switching to CO_2-based sterilization economically feasible and (v) it can be easily removed from the treated matrix by simple depressurization [9]. Recently, the sterilization with $scCO_2$ in different application fields such as those related to food [15–17] and biomedical products [18–21] was carried out. Reverchon et al. have shown that $scCO_2$ technology is very interesting in biomedical field to sterilize medical device like endoscopes which are very difficult to clean with common methods [19]. Indeed the main problems in the sterilization of these materials are connected with their complex geometry and with the nature of their constituting materials (such as metals, glasses, etc.) that present in their surface micro or nano spaces more easily accessible by gaseous or supercritical CO_2 [19]. Tarafa et al. reported the decontamination of both metallic and polymeric biomaterials removing lubricant oil from titanium surfaces and disinfecting poly(acrylicacid-co-acrylamide) potassium salt powders contaminated with *Staphylococcus aureus* (*S. aureus*) [22]. This work demonstrated that $scCO_2$ at relatively low temperature and moderate pressure can be used to remove contaminants from metallic surface and from hydrogel polyelectrolyte [22]. The same hydrogel used in the latter mentioned work was submitted to a CO_2 based cold sterilization by Jimenez et al. [18] after the contamination with *S. aureus* and *Escherichia coli*. These authors proved the effectiveness of CO_2-based technology in killing microorganism embedded in the hydrogel at 40 °C and 27.6 MPa after 4 h; furthermore, no significant changes in the hydrogel structure after exposure to CO_2 pure or modified by addition of H_2O_2 were observed [18]. Finally, a recent study reported the sterilization of corticosteroids for ocular and pulmonary delivery with $scCO_2$; these drugs used to treat allergies and ocular inflammation were successfully decontaminated and this result is of particular interest for the application in the case of aqueous suspension of steroids [23].

Bacterial spores are characterized by very resistant protective structures that make very difficult the sterilization of contaminated samples without damaging them. Thus the high temperature of some conventional sterilization techniques (used, for example, in the autoclaving process) can damage the scaffolds in an irreversible way. Therefore, it is very important to find out a sterilization method that does not alter the morphology and the chemico-physical properties of the polymer 3D matrix. Worth mentioning, it was previously shown that, usually, bacterial spores are drastically more resistant to treatment with pure CO_2 than vegetative bacteria unless high temperatures (80–100 °C) or extended treatment times (days) are used [8,24–30]. As an example, *Geobacillus stearothermophilus* and *Bacillus atrophaeus* spores strips were completely deactivated by $scCO_2$ at 300 atm in 25 min at 105 °C [8,26]. It was also observed that the presence of an additive as hydrogen peroxide, tert-butyl hydroperoxide, peracetic or trifluoroacetic acids can often allow to reach high removal of spores under more moderate conditions [8,31–33]. In particular Donati et al. obtained terminal sterilization of *G. stearothermophilus* spore loaded on paper strips after 4 h of exposure at 27 MPa and 40 °C to $scCO_2$ in the presence of H_2O_2 added at 200 ppm concentration [34].

In this work, we study the utilization of dense CO_2 to sterilize 3-D porous poly(L-lactic acid) (PLLA) scaffolds suitable for tissue engineering applications. The PLLA scaffolds were contaminated, both in surface and in the bulk, by the gram-negative bacterium *E. coli* and by spores of *Streptomyces coelicolor*; thus it was possible to analyze the effectiveness of the supercritical solvent as sterilizing agent of a three-dimensional massive structure with complex and tortuous pore geometry. We also studied the effect of the supercritical medium on the morphology and properties of the porous matrix. For this purpose scaffolds sterilized at different operative conditions such as treatment time and pressure were analyzed by DSC to observe possible changes in the crystallinity, while their morphology was observed by SEM micrographs. Finally, the effect of the treatment on the cell adhesion and growth was studied through cell cultures on CO_2 sterilized devices.

2. Materials and methods

2.1. Materials

PLLA (RESOMER^{TN} L 209 S) supplied by Boehringer Ingelheim Pharma KG, 1,4-dioxane (Sigma) and double distilled water were utilized to prepare the ternary solution for scaffold preparation. CO_2 used to perform sterilization experiments was supplied from Air Liquide (purity 99.998% vol/vol, water content ≤3 vpm). Ethanol (70% vol/vol) adopted in control sterilization tests and hydrogen peroxide (30% v/v in water) were both purchased from Aldrich.

2.2. Scaffold preparation and characterization

A homogeneous ternary solution composed by PLLA, 1,4-dioxane and water was prepared, with a polymer concentration of 4% w/w and a constant dioxane to water weight ratio of 87/13 w/w. The solution, initially kept at 60 °C, was poured into a cylindrical polypropylene sample holder (diameter, 14 mm; height, 30 mm). The temperature was then suddenly lowered to 30 °C (demixing temperature) for a well-defined demixing time (30 min), by pool immersion of the sample holder into a thermostatic water bath [35]. Then a quench by pool immersion in an ethyl alcohol bath at a temperature of −20 °C for 15 min was carried out in order to freeze the as-obtained structure. Finally, a freeze-drying step under vacuum at −28 °C for 72 h was performed for solvents removal from the sample by sublimation, in order to reduce the risk of damaging sample structure. By this methodology porous disk-shaped scaffolds with a diameter of 14 mm and a thickness of 2 mm were obtained.

2.2.1. SCF-sterilization apparatus

The experiments in dense CO_2 were carried out using the system shown in Fig. 1. This sterilization unit was constituted by a SFX 2-10 (ISCO) fluid extractor equipped with temperature controller and connected with a D260 syringe pump (ISCO). The pressure of the pump was controlled by a control system and the pressure in the zone near the supply valve of the extractor was monitored by a