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Research paper

Effects of supercritical carbon dioxide sterilization on polysaccharidic membranes for surgical applications

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ABSTRACT

Sterilization methods such as ɣ-irradiation, steam sterilization and ethylene oxide gas treatment can have negative effects on molecular structure and properties of polysaccharide-based biomaterials. In this perspective, the use of supercritical carbon dioxide ($scCO_2$) has been proposed as an alternative method for biomaterial sterilization. In this work, chemical, mechanical and biological properties of polysaccharidic membranes for surgical applications were investigated after sterilization by $scCO_2$. Four sets of sterilizing conditions were considered and SEC analyses were performed in order to identify the one with lower impact on the polysaccharidic matrix of membranes (alginate). Mechanical tests showed that the resistance of membranes was slightly affected after sterilization. Biological analyses proved the biocompatibility of the sterilized membranes both *in vitro* and in a preliminary *in vivo* test. Overall, this study points out that this sterilization technique can be successfully employed to achieve an effective and safe sterilization of polysaccharidic membranes for surgical use.

1. Introduction

Sterilization is a fundamental step for the manufacturing of biomaterials and implantable medical devices. This process is generally carried out through physical or chemical treatments that enable the removal of organic macromolecules and microorganisms in order to prevent infections in patients (Ahmed, Punshon, Darbyshire, & Seifalian, 2013; Delgado, Pandit, & Zeugolis, 2014). Sterilization procedures usually take place at the end of the manufacturing chain; several sterilization techniques are being used for this purpose and the choice of the most suitable method is typically done according to the type of biomaterial, the impact on material properties and the type of the potential contaminants (Delgado et al., 2014). The effects of sterilization on the properties of different types of biomaterials have been investigated in the literature (Dearth et al., 2016; Hofmann, Stok, Kohler, Meinel, & Müller, 2014; Park et al., 2012). In the case of polymer-based biomaterials, it has been reported that terminal sterilization techniques such as steam sterilization, ɣ-irradiation, and ethylene oxide gas treatment might have a strong impact on material characteristics (Lambert, Mendelson, & Craven, 2011; Murray et al., 2013; Phillip et al., 2013). For instance, ɣ-irradiation is well known to cause polymer degradation (Karajanagi et al., 2011; Lambert et al., 2011), while sterilization based on the use of ethylene oxide leads to the retention of toxic residues that can compromise the biocompatibility *in vivo* (Marreco, Moreira, Genari, & Moraes, 2004; Mendes, Brandão, & Silva, 2007). To overcome these drawbacks, the use of supercritical carbon dioxide ($scCO_2$) has been proposed as an alternative sterilization technique for biomaterials. The main advantages in the use of carbon dioxide (CO_2) for the sterilization of materials are related to its non-toxicity, non-inflammability and safety (Phillip et al., 2013) together with the possibility of easily removing it by depressurization and degasing. In its supercritical state, CO_2 has a liquid-like density ($0.9–1.0 \times 10^3$ kg/m^3) (Kanjickal, Lopina, Evancho-Chapman, Schmidt, & Donovan, 2008), gas-like diffusivity ($10^{-7}–10^{-8}$ m^2 s^{-1}) and viscosity ($3–7 \times 10^{-5}$ N s m^{-2}) and zero surface tension (Marreco et al., 2004), features that enable its penetration through materials. Methods based on the use of $scCO_2$ have been reported as effective for sterilizing medical products and bioactive materials (Donati et al., 2012; Herdegen et al., 2014; Pasquali & Bettini, 2008), and a proper combination between exposure time, temperature and pressure values was reported

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to influence the efficacy of the process (Spilimbergo, Dehghani, Bertucco, & Foster, 2003). The efficiency of sterilization can be improved through the addition of hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide or paracetic acid (Hemmer, Drews, LaBerge, & Matthews, 2007; Zhang, Burrows et al., 2006): these compounds can guarantee the inactivation of microorganisms, including bacterial endospores of different bacterial species (Russell, Rives, Pelletier, Bruce, & Walsh, 2013; White, Burns, & Christensen, 2006; Zhang, Davis et al., 2006). The use of such additives allows the employment of milder sterilizing conditions and shorter times of exposure (Qiu et al., 2009; Shieh, Paszczynski, Wai, Lang, & Crawford, 2009; Zhang et al., 2007). Several papers proved that $scCO_2$ combined with low amounts of additives is effective in bacterial spore killing (Checinska, Fruth, Green, Crawford, & Paszczynski, 2011; Park, Choi, Kim, & Kim, 2013; Shieh et al., 2009), suggesting that this emerging technique can be successfully employed for biomaterials sterilization. Despite promising results regarding the use of $scCO_2$ for biomaterial sterilization have been obtained, little work has been done in evaluating how this sterilization method affects materials features. For instance, it has been shown that heat-sensitive biomaterials might undergo degradation upon exposure to high temperature and pressure (Zhang et al., 2007); moreover, the use of compounds such as H_2O_2 may cause polymer oxidation and depolymerisation (Li et al., 2010; Zhang et al., 2007), thus modifying the chemical and physical properties of biomaterials. With regard to the biocompatibility of sterilized biomaterials, both *in vitro* and *in vivo* evaluations are needed to assess the possible cytotoxic effect that they can exert in a biological system (Bernhardt et al., 2015). Thus, a detailed characterization after sterilization is required to evaluate possible modifications of the sterilized biomaterial.

Recently, Travan et al. developed a polysaccharide-based membrane for biomedical applications (Travan et al., 2016); this membrane was based on a mixture of alginate and hyaluronan (HA), the former representing the physical matrix, the latter acting as the bioactive component once released at the wounded site, given the ability of HA to stimulate wound healing (Dicker et al., 2014; Price, Myers, Leigh, & Navsaria, 2005; Voigt & Driver, 2012). This membrane can be applied when the closure of surgical wounds (*i.e.* intestinal anastomosis) needs to be stimulated in order to prevent post-operative complications. In case of intestinal anastomosis, the membrane can be wrapped around the sutured site, in order to support and stimulate tissue regeneration. In this work, we have analysed the impact of $scCO_2$ sterilization (in the presence of H_2O_2) on several features of these membranes; mechanical, chemical and biological properties were investigated and a preliminary *in vivo* test was performed to evaluate the biocompatibility of the sterilized membranes on intestinal tissue.

2. Materials and methods

Sodium alginate from *Laminaria hyperborea* (Alginate Pronova UP LVG, molecular weight, MW ~ 120,000; fraction of guluronic G residues, $F_G = 0.69$; fraction of guluronic diads, $F_{GG} = 0.59$; number average of G residues in G-blocks, $N_{G>1} = 16.3$) was kindly provided by Novamatrix/FMC Biopolymer (Sandvika, Norway). Sodium hyaluronate (hyaluronan) Pharma grade (HA MW ~ 240,000) was kindly provided by Sigea S.r.l. (Trieste, Italy). Calcium carbonate ($CaCO_3$), D-Gluconic acid δ-lactone (GDL), glycerol, hydrogen peroxide (H_2O_2), LDH (lactate dehydrogenase)-based TOX-7 kit, Hanks' Balanced Salt solution (HBSS), Iodure Potassium (KI), Sodium Hydroxide (NaOH), Ammonium Molybdate ($(NH_4)_2MoO_4$), Sodium Chloride (NaCl), Potassium Hydrogen Phtalate ($C_8H_5KO_4$), hematoxilyn and eosin were purchased from Sigma Aldrich. Primary human dermal fibroblasts (HDFa) were purchased from Invitrogen™ Life Technologies. The cells were cultured in Medium 106 (Gibco™) supplemented with Low Serum Growth Supplement (Gibco™).

2.1. Membrane manufacturing

All membranes were prepared according to the procedure reported by some of the authors of this paper (Travan et al., 2016). Briefly, alginate or a mixture of alginate and HA were dissolved in deionized water (final concentration = 15 g/L of each polysaccharide) and glycerol (final concentration = 5% v/v) was added as a plasticizer. Suspension of $CaCO_3$ (final concentration = 20 mM, corresponding to $[Ca^{2+}]$ 20 mM), and GDL (final concentration = 40 mM) were added to the mixture that was poured into rectangular moulds for the *in situ* gelation of the solution. Subsequently the hydrogels were freeze-dried using a Single-ChamberFreeze-Dryer (Christ Alpha 1–2 L Dplus).

2.2. Sterilization of membrane with gaseous H_2O_2 and ɣ-radiation

Membranes were sterilized by ɣ-radiation (<25 KGy) and gaseous H_2O_2. In this latter case, sterilization was carried out through a custom-made equipment developed by I.E. "Impuls" (Gdansk, Poland) using the following parameter: 250 ppm gaseous H_2O_2, 30% −50% humidity.

2.3. Membrane sterilization with $scCO_2$

Membranes (3 cm × 5 cm) were exposed to $scCO_2$ under controlled conditions in 1 L supercritical CO_2 pilot reactor custom made for Rescoll (SEPAREX). Four sets of conditions were employed.

	Pressure (bar)	Temperature (°C)	Initial content of H_2O_2 (ppm)	Exposure time (hour)
Set 1	270	40	200	1
Set 2	270	40	1000	1
Set 3	270	40	200	3
Set 4	270	40	1000	3

The high pressure vessel was disinfected with ethanol prior to use. H_2O_2 was added to the vessel by using a sterile medical cotton pad. A standard CO_2 cylinder was used and the gas was pressurized by a high-pressure syringe pump (SEPAREX, P200) equipped with a EUROTHERM Nanodac heating unit. The reactor temperature was controlled *via* a PT100 sensor (TC Direct). The internal pressure of the reactor was controlled by a transducer (STS) connected to a pressure control unit (EUROTHERM Nanodac).

2.4. Mechanical tests

The membranes were cut in dog-bone shapes according to ASTM D638-10 standards (type 1 samples) and their mechanical properties were studied using a Universal Testing Machine (Mecmesin Multitest 2.5-i) equipped with a 100 N load cell. Tensile tests were performed in uniaxial configuration at a constant crosshead speed of 5 mm/min. Tensile stress was calculated dividing the load by the average original cross sectional area in the gage length segment of the specimen. Young's Modulus (E) was calculated as the slope of the linear portion in the stress-strain curve, considering the deformation range of 1%–3%. For each condition, five replicates were used.

2.5. Membrane dissolution

Sterilized membranes (3 cm × 3 cm) were transferred in a dialyzing tube (MW cut-off 10.000) and the dialysis was carried out against aqueous HCl 0.1 M (4 shifts) at 4 °C, since HCl enables the