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A novel supercritical CO_2-based decellularization method for maintaining scaffold hydration and mechanical properties

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ABSTRACT

Decellularized tissues are commonly utilized as tissue engineering scaffolds. Decellularization by extended exposure to aqueous detergents can damage the microstructure or deposit cytotoxic residue. Supercritical carbon dioxide ($scCO_2$) has been proposed for decellularization, but reportedly causes dehydration and scaffold embrittlement.

Presented herein is a novel decellularization method that preserves matrix hydration state and mechanical properties. Over 97% of the water in porcine aorta is maintained by presaturating $scCO_2$ with water; however, complete decellularization was not attained by any process utilizing only $scCO_2$. Instead, a novel hybrid method is presented that combines a brief (48 h) exposure of tissue to aqueous detergent, followed by washing with $scCO_2$ (1 h). The hybrid method fully decellularized the tissue, as confirmed by histology and DNA quantification (<0.04 μg DNA/mg tissue). This hybrid treatment was faster than the standard method (2 days compared to 4–7 days), while preserving tissue structure and mechanical properties.

1. Introduction

Over 8000 Americans die annually while awaiting an organ transplant, and currently over 120,000 Americans are on the national transplant waiting list. Furthermore, the average transplant wait time is several years [1]. One way to address this problem is the implantation of artificial tissues and organs created by tissue engineering (TE). This could drastically reduce wait times and alleviate the current dearth of available organ donors. However, tissues and organs are extraordinarily nuanced and complicated structures, presenting numerous requirements for creating effective biomimetic materials.

Whether derived from synthetic or natural materials, TE scaffolds must be sterile, porous, mechanically strong, biocompatible, and of appropriate stiffness and surface chemistry for their specific application [2]. Additionally, the scaffold fabrication process may introduce several structural and biochemical deficiencies, including loss of mechanical strength, loss of surface activity, denaturation of extracellular matrix (ECM) proteins, scaffold dehydration, and residual cytotoxicity of some solvents, detergents, and/or crosslinking agents [3]. All of these challenges require continual development of novel and innovative scaffold fabrication methods.

Additionally, TE scaffolds must direct cell proliferation and differentiation during tissue growth. This is a particular strength of naturally-derived biomaterials, which have recently been shown to promote constructive remodeling during tissue growth [4,5]. Decellularized ECM has also been shown to elicit an anti-inflammatory immune response, which may be related to a reduced risk of rejection [6,7].

Decellularization is accomplished using a variety of techniques, including physical [8], chemical [9], and enzymatic treatment methods [10]. Treatment with aqueous detergents, such as sodium dodecyl sulfate (SDS), is most common [11,12]. Detergents lyse cell and nuclear membranes, which can lead to thorough cell removal, but also denature proteins and can disrupt glycosaminoglycans (GAGs), growth factors, and ECM ultrastructure [13]. Because of these hazards, it has become common in some tissues, such as blood vessels, to treat with detergents at very low concentrations over multiple days or even weeks, avoiding damage to ECM while eventually removing all cells [14]. Additionally, some decellularization protocols may be relatively brief, but require prolonged wash cycles to remove residual detergent [15]. Though these approaches can be effective, novel methods are desired to decellularize tissues as effectively but with shorter treatment times and without using harsh chemicals or solvents for long periods.

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One relatively unexplored method worthy of consideration is using supercritical carbon dioxide ($scCO_2$) instead of aqueous- or alcohol-based solvents. $scCO_2$ is non-toxic, non-flammable, and relatively inert; it has desirable solvent properties and a mild critical temperature (31.1 °C), making it viable at physiologic temperatures [16]. Supercritical CO_2 has been utilized in numerous biomedical applications, including extraction of biologically relevant molecules [17,18], pasteurization [19–21], and sterilization of synthetic [22–24] and natural biomaterials [25,26]. $scCO_2$ has been used extensively to fabricate TE scaffolds from synthetic biomaterials, such as by polymer foaming [27–29], without any significant loss of scaffold bioactivity [30]. Though supercritical drying of natural TE scaffolds has been achieved [31], little research has been undertaken on using $scCO_2$ to fabricate TE scaffolds directly from natural biomaterials, such as by decellularization.

By virtue of its favorable transport properties compared to water, a $scCO_2$ decellularization treatment could offer considerably faster treatment, on the order of hours instead of days. Eliminating detergent use would also reduce damage to the ECM and cytotoxicity associated with residual detergent. In 2008, Sawada et al. presented a study on supercritical CO_2 decellularization [32]. They reported adequate DNA and cellular removal, but also extensive extraction of volatile substances during treatment, primarily water [33]. Dehydration caused hardening of the tissue and subsequent scaffold embrittlement, potentially endangering the viability of the scaffold. This raises a significant obstacle to progress in the field.

Other decellularization protocols that utilize ethanol as the decellularization solvent have reported similar tissue dehydration [34], so the observed extraction of water and volatiles during treatment with $scCO_2$ and ethanol is not surprising. In fact, water extraction is very similar to critical point drying, which is commonly used in tissue engineering [35] and other applications, such as electronics processing [36] and scanning electron microscopy [37]. However, for decellularization it is desirable to prevent drying entirely. We hypothesize that tissue dehydration caused by $scCO_2$ treatment can be significantly reduced or even eliminated by presaturating $scCO_2$ with water and other biological volatiles prior to treatment. Validation of this hypothesis is the necessary first step before proceeding to developing a process to decellularize xenogeneic tissue for tissue scaffolds.

Our broad aim is to develop an effective and efficient decellularization method that utilizes $scCO_2$ to reduce treatment time, which will enable further development of $scCO_2$-based tissue engineering and decellularization processes. The objectives of this work are as follows: (1) to demonstrate how to maintain the hydration state of the native tissue in the presence of $scCO_2$, and (2) to present a hybrid $scCO_2$/detergent treatment that decellularizes the tissue more quickly and as effectively than a standard detergent treatment while maintaining hydration and mechanical properties.

2. Materials and methods

2.1. Apparatus development and validation

To prevent water extraction from porcine tissue it is necessary to first achieve dynamic thermodynamic equilibrium (*i.e.* complete saturation) between $scCO_2$ and water. The saturated $scCO_2$ phase is subsequently suitable for treating a TE matrix. The first experimental objective was to ensure that the $scCO_2$ was being fully saturated during the mixing process. Achieving this goal was critical before attempting to decellularize a tissue.

A schematic of the presaturation apparatus is shown in Fig. 1. The apparatus contained valves and fittings rated for high pressures up to 68.9 MPa (2) (High Pressure Co., Erie, PA). Liquid carbon dioxide (1) (bone-dry grade with siphon tube, 99.8% purity, Praxair Inc., Danbury, CT) was compressed in a chilled syringe pump (3) (500 HP Series, ISCO Inc., Lincoln, NE) and slowly bubbled into the presaturation chamber (5) (Waters Corp., Milford, MA), where 10 mL water was previously added. In this chamber $scCO_2$ and the water additive were stirred vigorously until reaching thermodynamic equilibrium (about 15 min). At this point, flow was introduced into the 10 mL treatment chamber (6), which contained the matrix material. Constant flow was maintained by the syringe pump at 1 mL/min for the desired treatment time (usually about 1 h). At the end of that time, a manually-operated pump (8) (Pressure Generator 62-6-10, High Pressure Equipment Co., Erie, PA) connected to the treatment chamber was used to depressurize the system at a controlled rate of 0.345 MPa/min (50 psi/min) after treatment.

Validation of the apparatus was demonstrated by using a cold trap to collect dissolved water in the effluent after the back-pressure regulator. Complete thermodynamic equilibrium between $scCO_2$ and water was achieved at flow rates of 5 mL liquid CO_2/min and below, as measured at the syringe pump. Validation data are presented in Fig. S1. At flow rates 5 mL/min and below, the effluent water mole fractions approach the equilibrium limit. As the flow rate increases, the observed mole fraction decreases, indicating failure to equilibrate. CO_2 flow rates of 1 mL/min were used for the remainder of this work.

2.2. Biomaterial selection and preparation

To further investigate the presaturation hypothesis, we utilized both a synthetic biomaterial (a hydrogel) and a natural tissue, porcine aorta. The hydrogel was poly(acrylic acid-*co*-acrylamide) potassium salt (Sigma-Aldrich, St. Louis, MO), a hydrogel used previously to establish the ability of $scCO_2$ to achieve sterilization within a porous matrix [22]. Hydrogel powder was hydrated in excess water at 4 °C for 24 h. Excess water was removed from each hydrogel specimen by light vacuum for 15 min with a Buchner funnel. The hydrogel was next blotted onto a nylon filter and sealed inside the treatment chamber prior to exposure to high pressure CO_2. The weight of each gel was approximately 0.2 g.

Porcine aorta was obtained from a local slaughterhouse and the surrounding fatty tissue was removed. The aortic tissue was cut into thin rectangles (approx. 3 cm × 2 cm) and stored in phosphate-buffered saline (PBS) at 4 °C for up to 48 h prior to use. Each tissue specimen was dried for 15 min under a light vacuum using filter paper and a Buchner funnel; this removed free saline prior to weighing and treatment. Extensive drying in a vacuum oven (37 °C, 38.1 cm Hg vacuum) was used as a negative control; changes in tissue mass were recorded after 1, 2, 3, 6, and 24 h. The treatment ratio (i.e. total mass of CO_2 per unit mass of hydrated material) and other conditions used (including temperature, pressure, and depressurization rate) were chosen to be analogous to the conditions used by Sawada et al. to allow for comparison [32].

2.3. Dehydration of model matrix materials

All treatments were performed using the apparatus shown in Fig. 1. In these tests, a hydrated biomaterial (hydrogel or porcine aorta) was weighed, then treated with either dry $scCO_2$ or presaturated $scCO_2$. After treatment, the biomaterials were weighed again, and changes in mass were recorded. Two treatments were conducted on each biomaterial: one using dry $scCO_2$ (no water in presaturation chamber) and the other using $scCO_2$ presaturated with water. All treatments were performed at 13.8 MPa (2000 psi). The temperature was held constant at either 37 °C (ρ_{CO2} = 0.769 g/mL) or 50 °C (ρ_{CO2} = 0.665 g/mL, for hydrogel only). Four replicate treatments were made at each temperature. All biomaterials, regardless of initial mass, were subjected to a treatment ratio of 60 min of $scCO_2$ flow per 0.2 g gel or tissue.

2.4. Standard decellularization with SDS

For decellularization studies, porcine aorta was obtained from a local abattoir, rinsed in PBS and cut into ring-shaped sections